

What Gorbachev must do now

The unexpected return of Mr Mikhail Gorbachev is heartily to be welcomed, even if his spell in Moscow will be relatively brief. But the rest of us have things to do as well.

THERE is a story making the rounds that Mr Mikhail Gorbachev's somewhat subdued appearance at the G7 meeting in London last month resembled that of an applicant for a research grant whose application had been 'approved, but not funded'. But the truth is not quite like that. The application was not really approved, but (in the argot of the grant-making business) the money-bags (the governments of seven of the world's largest countries) decided that the applicant had promise and advised him to rework his application and to resubmit rather than risking outright refusal. Now, both the applicant and the grant-makers will have to think again, and quickly. Before the first snows fall in Moscow, the present euphoria will have been overlaid by images in Soviet people's minds of the empty shops and by the prospect of another long winter of deprivation. What should the West now do?

The place to start is with a full-throated recognition of our great good fortune at the way things have turned out. If the coup against Gorbachev had succeeded, not only would the people of the Soviet Union have been thrown back under repression, but most of the welcome developments of the past few years would have been put in hazard.

Risks averted

The Cold War would have been reopened. The three important arms control agreements of the Gorbachev period (on intermediate nuclear missiles, conventional forces in Central Europe and the reduction of strategic missiles agreed only in July) would probably have been judged in Moscow to be incompatible with the coup-makers' declared aim of "making the motherland great again"; certainly the provisions for on-site inspection which they include would have been scorned. It is even possible that the determination of the Baltic states to be independent would have drawn the West into direct conflict with the rump of the Soviet Union. Almost certainly, the states of Central Europe now independent of the Soviet Union would have been given serious cause for alarm about their future. Does not deliverance from this awful prospect require some tangible recognition by the industrialized West?

Luckily, Gorbachev is now in a position to rewrite his application so as to command the support of those in the West whom he has been addressing. The collapse of the coup will have discredited not only those who mounted that foolish enterprise, but opponents of reform within the

Soviet Union more generally. What should he do?

Opportunities

After possibly damaging hesitation, Gorbachev has done what he should have done months ago: he has quit his post as general secretary of the Communist Party. There has always been an intolerable conflict of interest between the advocacy of the interests one party expected of its general secretary and the supervision of a developing constitutional and legal framework within which that party's privileges must be attenuated and dissent from its opinions allowed, even encouraged. One of the abiding follies of the whole Soviet enterprise is that the party has been placed so as to second-guess the government, just as party cells in laboratories have second-guessed the decisions made by research directors and others. It is in everybody's interest that this absurd scheme seems now on the way out.

There is much else to do. Although it is still not clear what will be the consequences of the declarations of independence by republican governments such as the Ukraine and Byelorussia (not to mention the Baltic states and Georgia), most of the 15 republics will have to hang together for a while; they do not have the means to become truly independent states, while 70 years of central planning have skewed their development away from the natural course. At least for a time, they will need some elaboration of the Union treaty whose intended signature on 20 August seems to have helped to precipitate the abortive coup. That has some of the right ingredients, but not enough of them. The crucial weeks ahead are also those in which the machinery for the government of Soviet science over coming decades will be decided.

The principle that the Union should be a federation of sovereign states ceding responsibility for external defence and monetary management to the centre is all very well, but leaves unanswered most of the questions with which the European Communities are still struggling in their negotiations on political and monetary union. But the Soviet situation is even more complicated; the centre would hold and control the vast stock of nuclear weapons, for example, but the Union treaty as it stands provides that decisions on their deployment or, shudder the thought, use, would be made jointly by the republics and the union government. Would that be workable? And safe? And it is not clear how less prosperous members of the union



would be helped to bridge economic disparities with richer members. Gorbachev needs a better union treaty, ideally by tomorrow if not sooner.

There is a vast amount of constitutional, legal and social work to do. Thus the coup-makers' complaint that the Soviet Union has become a criminal society is sadly correct. It is strange, but not really surprising, that the country has been transformed in six years from one of the most law-abiding places in the world to one of the least. The explanation has three parts. First, the enforcement of the law has rested with the KGB and the Interior Ministry's police, at once feared and despised for arbitrariness, and with a judicial system over-dependent on the enforcement agencies. Second, custom and practice in the Soviet Union have so consistently endowed some classes of place-holders with privileges as to blur the distinctions between proper and corrupt behaviour. Third, general impoverishment has made it seem that crime must pay.

The central government is most directly in control of the petty privileges enjoyed by public servants (members of the Academy of Sciences conspicuous among them). Calling a halt to this shabby trade would have an instantaneously beneficial effect. Beyond that lies only the long haul of the social and administrative engineering required to make a judicial system that seems fair to those who live within it. It will be difficult to shake off the tradition that most police are secret police, and to create penalties that match the crimes they are meant to punish.

Luckily, in the aftermath of the coup that failed, the Soviet Union's economic difficulties may prove to be less serious than Gorbachev has found so far. There is no shortage of recipes for reform. The difficulty so far has been that of settling for one of them — and then of sticking to it. It seems to be agreed, at least among the republics ready to sign the Union treaty, that there should be an all-Union market in goods and services, but the treaty seems also to contradict that plan by specifying means by which salaries and other rewards are fixed. But there is a sense in which any plan will be better than none, which is the present state of affairs.

Already there has been a move to decontrol the prices paid by Soviet enterprises to each other (which explains much of the decline of output in the past two years) but the government has so far shrunk from giving up price control for the basic necessities of life at the retail level. The time has come to bite that bullet. But how, without causing intolerable hardship among already impoverished people? Most simply, by abolishing price controls on essential foodstuffs and then flooding the market with such a surplus that prices do not rise much, at least until the winter ends. As luck will have it, there are ample stocks in the warehouses of the West. Gorbachev needs a way of working this trick, which is where G7 should most urgently think again. It should offer the Soviet Union unlimited credits to buy food (at market prices, so as not further to upset the General Agreement on Tariffs and Trade) with the promise that debts thus incurred would be written off if there were a sensible economic plan in place

by, say, the beginning of next year. The cost might be \$5,000 million, but it would be well worth it.

What should be the criteria of success? To say that there must be a free market is not sufficient. Market prices can always make supply and demand balance, but the prices would still be unreal while state regulations on employment remain and while the pitifully inadequate transport system fragments the market geographically. Worse still, in spite of the 70 years during which Soviet governments have boasted of their solicitude for individuals, the social security system does not adequately cater for the unemployed or for pensioners. Nor is there the inevitable other side of that coin — an equitable system of personal taxation. Not all this can be in place in a few months. But a resolute plan could be monitored from outside.

What will happen to science under this new order? Under the draft Union treaty, the centre will remain responsible for the supply of weapons (and thus for defence research) as well as for space research and nuclear energy, but the centre and the republics would be jointly responsible for fundamental research and its application. Quite how this would work in practice is not spelled out, but the lessons of the past point to several steps that should be taken. The old all-Union Academy of Sciences should, for a start, be split into a regular academy like everybody else's and a research organization better able to manage the host of laboratories scattered about the vast Union, but mostly in the Russian Republic. Second, the universities which have been consistently deprived of opportunities for research should be taken in as partners in the research enterprise. But it is also clear, in the Soviet Union as elsewhere in Eastern Europe, that the state at present supports many more researchers than it needs — or can afford. Nobody should be surprised if the growing army of the unemployed in the Soviet Union comes to include a million or so out of work researchers.

Luckily, the West can help — and it should. The immediate need is to secure the population against the hardships of the winter ahead. Beyond that, the need will not be for huge sums of public money, but for a framework within which private investment from outside can be made to seem secure and profitable. Luckily, the Union treaty would allow separate republics to make their own arrangements for economic development in this way; they will have an incentive to compete with each other in making their arrangements attractive. The snag that will quickly become apparent is that even an efficient Soviet manufacturing industry would have few opportunities for selling its goods abroad. Too many markets are either closed to it, or protected by tariffs. That is why the G7 countries should spend the winter working at the mechanisms by which this previously isolated part of the world can be made part of the global economic community. (The states of Europe have a more urgent task — that of opening the European Communities towards the East.) Gorbachev may have a lot to do in the months ahead, but so have the rest of us. □

Hungarian science faces sweeping reforms

- Universities will switch to Western model
- New competitive grant system to prune staff

Budapest

In the Soviet-style government laboratories and universities of Hungary, 30,000 researchers will soon be grappling with the ingredients of a brave new world — competitive grants and a graduate research programme. These new features will usher in what officials here say will be the biggest shake-up of Hungarian science since the Communist takeover.

Starved by plummeting government funding and the collapse of private research-based industry, it might seem that Hungarian universities and research institutes could have found a better time to reinvent themselves. But on the other hand, the changes may hold the best promise for saving as much as possible of what's good in Hungarian science.

For an emerging democracy, change — even great change — is to be expected. But Hungary's coming research revolution is due as much to the scientific enterprise's crippled condition as it is to new ideology. In the last three years, government research funding has dropped by 25 per cent relative to the country's economy, from over 2.3 per cent of gross domestic product in 1988 to less than 1.7 per cent in 1990. Compared to the US dollar, government funding has dropped by about half — from some \$200 million (in constant 1991 dollars) in 1980 to \$100 million today.

As hard as this has hit the 3,200 researchers in the 52 institutes of the Hungarian Academy of Sciences, some of whom have been unable to buy test tubes and reagents for over a year, it has been worse for the universities.

Academic science in Hungary has been on a decline since 1947, when the universities were stripped of their best researchers by the government to set up the research institutes of the academy, part of a political ideology that sought to exercise state control of research. At risk of being shut out of research altogether, the universities turned to applied science and technology in an attempt to woo industrial support.

Unfortunately, that worked only too well. By the mid 1980s, over 80 per cent of university research and development (and 40 per cent of total university revenues) was funded by industry. But the industrial sector had its own problems. When, in the latter half of the last decade, the Soviet economy failed, the factories in Central Europe that fed it crumbled also.

Industrial support fell from 76 per cent of total Hungarian science funding in 1980, to just 47 per cent in 1989 (the last year for which figures are available). Recent years have been even worse. This spring, the largest Hungarian electronics company, Videoton, which had relied largely on the Soviet defence industry, announced that it was unable to continue operations. Over 1,000 scientists and engineers may lose their jobs.

Universities have therefore been left with departments working on technology that no one outside of Eastern Europe wants and that no one inside Eastern Europe can afford. They have been out of basic research so long that they have little chance of getting back in on their own, without major help from government.

That is just what the new changes in the Hungarian university system are meant to be — a way to get science back into higher education. Under a new plan — called the Athenaeum Initiative — expected to take effect next year, Hungarian universities are about to get the authority to issue Western-style science PhDs for the first time since the Second World War. At present, the power to issue scientific degrees rests with the granting committee of the education ministry.

Under the Athenaeum Initiative, universities will train graduate students through a four- to five-year, PhD-equivalent science degree. A higher degree, awarded on the basis of scientific achievement and expected to be called a Doctorate of Sciences, will be issued by the Hungarian Academy of Sciences. To further the mixing of science and education, academy researchers will be expected to teach part time, and university professors will be given leave to do research at government institutes.

Along with the reform of graduate education, science officials are also putting into place a new competitive grants programme that will replace the incestuous 'old boys network' that has dominated Hungarian science for four decades. This spring, all Hungarian researchers got a chance to nominate members to the new committees that will evaluate grant proposals for the academy as well as for the Hungarian Research Fund, known as OTKA.

Some 6,000 scientists — almost 30 per cent of the 22,000 full-time scientists now working in Hungary — were selected to serve on the committees and will now get

their first opportunity to rank research proposals independently of party affiliations and institutional loyalties. The committees will be split up into dozens of small discipline-specific subcommittees, much like the study sections of the US National Institutes of Health.

"Previously the idea was to spread the money more or less equally to the [academy] institutes, to keep them all going," says Gèza Bittsàszky, head of the government's secretariat for science policy. "Now the funding will be based on quality. Good researchers will get more than before, and poor researchers will get less and will eventually have to leave. I am convinced that half of the people [in the academy laboratories] must find jobs in other areas. Instituting a competitive grants process is a good way to do this."

Officials hope that breaking up the bloated academy research network will have another advantage — it can help turn Hungary away from second-rate technology and back to the basic research that it can still do well. Like the universities, many academy research institutes turned to industry contracts — and more applied science — to make up for the shrinking government funding of the last decade. And like the universities, they found themselves cut off from both markets and leading-edge basic research when industry collapsed. Some of these institutes — among them the Central Research Institute for Physics and the Computer and Automation Research Institute — have already begun major reforms that are expected to shed applied science groups and eventually cut research staff.

"The government does not give up on the support of technology and technical development," says Hungary's official reform agenda. "The role of the state will be narrowed down in this field, however, and will be more marked in strategic areas where there is not enough competition yet." Officials say that chemistry and agricultural biotechnology are fields where Hungary has been able to keep up with international standards and can still compete.

Behind all the changes is the underlying hope that bringing Hungary's science back into the real world may encourage some of its young researchers to stay in the country. In any one year, some 12 per cent of all Hungarian researchers are working abroad; about one-quarter of those have been away for at least five years. This has hit young researchers the hardest; in the past several years the number of scientists in their twenties and thirties has fallen by 30 per cent in some fields. Seven Nobel laureates are proof enough that Hungary can produce good scientists. Officials hope the reforms will convince some of the departed researchers that it is now safe to come back.

Christopher Anderson

Wanted: Home-grown industry

Ithaca, New York

In the National Nanofabrication Facility at Cornell University, a recently delivered focused-ion-beam machine sits on the floor, so new that it has not yet been assembled. The million-dollar instrument will help the Cornell centre remain at the forefront of the growing field of nanotechnology, giving the researchers who work here precise control over the chemical composition of the microscopic devices they construct.

But the facility won't be buying any more machines from Microbeam, the small California company that manufactured the state-of-the-art ion-beam device. Microbeam has closed, and the only suppliers of comparable equipment are in Japan.

This episode typifies a general decline of US companies that make the equipment used in nanofabrication — the production of devices on the scale of nanometres. This trend has already created serious problems for the US microelectronics industry, and some US researchers fear that the growing dominance of Japanese equipment manufacturers could threaten the current US lead in basic nanofabrication research and eventually even undermine US competitiveness in future nanotechnology-based industry.

These concerns will be aired at a National Science Foundation (NSF) workshop in Washington next month, when up to 100 researchers from academia and industry will consider the future for nanotechnology in the United States. The immediate issue is NSF's support for the Cornell facility — the \$2-million-a-year, five-year grant from the foundation is up for renewal at the end of the 1992 fiscal year. But the broader threats to US research and industrial competitiveness are bound to surface, particularly now that the powerful Japanese Ministry of International Trade and Industry is launching a ten-year nanotechnology project worth hundreds of millions of dollars (see *Nature* 352, 650; 22 August 1991).

Harold Craighead, director of the nanofabrication facility, says the experience with Microbeam is not unique. Several times in recent years, the Cornell centre has taken delivery of equipment from US companies that were either going out of business, or being bought out by their competitors. "Some of that's normal shakeout for these companies," Craighead says, but he believes the US industry is in real decline. Most of Craighead's colleagues agree with this assessment.

US nanotechnologists don't agree, however, about the implications of the growing domination of Japanese equipment manufacturers. Greg Blonder, from AT&T Bell Laboratories, does not expect

many problems. Japanese suppliers, he says, are usually willing to provide cutting edge equipment to US laboratories — provided that researchers take the time to develop close links with Japanese companies. "You have to be careful that you try hard enough," Blonder says.

But Mike Isaacson, a Cornell applied physics professor who sits on the executive committee that oversees the nanofabrication centre, fears that Japanese manufacturers will place still-developmental nanofabrication equipment in industrial or university laboratories in Japan for testing, giving researchers there up to two years' head start with new technologies over their US counterparts. George Hazelrigg, an NSF official responsible for nanotechnology, says there have already been a number of examples of Japanese equipment manufacturers giving researchers in their own country preferential access to new devices.

Whatever the implications for basic research, most US nanotechnologists agree that the dominance of the Japanese equipment industry will give Japan an important advantage five years or more from now, when some of the applications for ultra-small structures now under development begin to be commercialized. Blonder, despite his confidence that Japanese equipment makers will not discriminate against US researchers, concedes that the story may be very different when it comes to providing the manufacturing equipment for a nascent nanotechnology industry.

The problems faced by US nanofabrication equipment companies are closely linked to the state of the US semiconductor industry. Nanofabrication is essentially a spin-off from the technology used in semiconductor production, and the US semiconductor-processing-equipment industry has suffered a rout in recent years: According to a survey by VLSI Research, a company that tracks the industry, the US share of the world market fell from 69 to 43 per cent between 1983 and 1990, while that of Japanese companies rose from 25 to 49 per cent.

Many industry observers blame the failure of US chip manufacturers to invest in or even consult closely with the home-grown equipment industry, in stark contrast to the Japanese tendency for semiconductor producers to form strategic alliances with their equipment suppliers. The Sematech consortium of US semiconductor manufacturers is now trying to address this problem, and its success in rescuing the US semiconductor-processing-equipment industry may be essential if any future US industrial push in the broader field of nanofabrication is not to be left in the starting blocks.

Most observers say that it will be several years before Sematech's success can be judged. "It took us ten years to get to the sorry state that we're in, and it'll take us ten years to get out," says former NSF director Erich Bloch, now with Counsel on Competitiveness. A sobering thought, as his former organization sits down to consider its future support for nanotechnology.

Peter Aldhous

MEDICAL RESEARCH

UK ethics guidelines

THE UK Department of Health has published its first guidelines for the vetting of proposals for research involving human subjects in the National Health Service (NHS). Under the guidelines, each NHS District Health Authority must set up an ethics committee with eight to twelve members, including at least two lay representatives, one of whom should be the committee's chairman or vice-chairman. Health minister Virginia Bottomley last week said that this provision should ensure that lay members are not marginalized by medical professionals on the committees.

The new guidelines also lay down some basic ground rules for research on human subjects in the NHS. Patients should be clearly informed that their participation in research projects is entirely voluntary — given the unequal relationship between doctor and patient, there have been fears that patients might agree to participate in research projects under the mistaken belief that their treatment could suffer if they do not do so. Written consent should be obtained from all subjects, both patients and healthy volunteers. And where this is impossible, for example in projects involving the mentally incapacitated, ethics committees should pay careful consideration to the risks and benefits of the research. The guidelines also demand that researchers keep confidential the personal health records of volunteers enrolled in research projects.

The government guidelines follow, and are largely based upon, the efforts of the scientific community to police itself. A number of organizations, including the Medical Research Council, the Royal College of Physicians and the Association of the British Pharmaceutical Industry have already published their own guidelines, and over the past five years, most District Health Authorities have appointed ethics committees. The research council has for several years refused to fund projects involving human subjects until they have been approved by an ethics committee.

The local ethics committees will also review research proposals involving fetal tissue, *in vitro* fertilization, or the use in NHS hospitals of recently deceased cadavers.

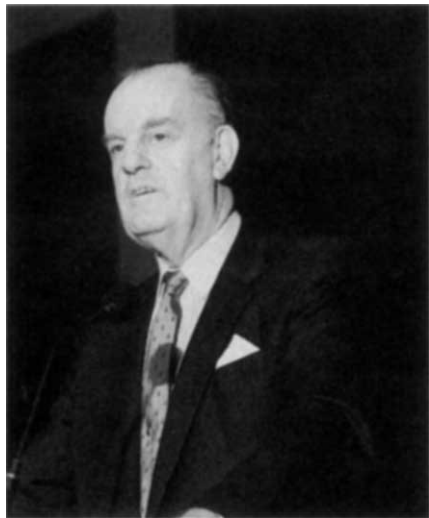
Peter Aldhous

No bliss in ignorance

Plymouth, Devon

BRITAIN may be spending enough on scientific research but the "abysmal ignorance" of ministers and civil servants means the country is failing to capitalize on this investment, Sir Denis Rooke, president of the British Association for the Advancement of Science, told the annual meeting in Plymouth this week.

"You only have to deal, as I have over many years, with civil servants and ministers to realise the depth of their igno-



Sir Denis lambastes civil servants.

rance of scientific affairs. It is absolutely abysmal, much greater than my ignorance of the mechanisms of politics."

Sir Denis told delegates not to be side-tracked into equating Britain's relative failure in innovation with inadequate funding of the science base.

"A huge improvement in industrial innovation could take place using the fund of scientific knowledge already possessed," he said. "It is in the determined and innovative application of science and related technology that we in Britain are lagging today as a nation."

The answer, according to Sir Denis, an engineer who spent 40 years at the national utility British Gas, crowning his career as the chairman who steered the company through privatization, is to have more research organized directly by industry. This could either be in company labs or sponsored at universities.

UK companies should also scour the world for relevant scientific and technical advances, in the way the Japanese do. "The leaders of Japanese business tell me they send many of their brightest young scientists to the UK to pick up the latest scientific developments."

"It is on that intelligence that they build, creating profitable new products, well designed and efficiently manufactured," Sir Denis said.

Nuala Moran

HUMAN GENOME MEETING

The price of success

London

DOUBT clouds the future of the Human Genome Organisation (HUGO) gene mapping conventions after a tentative restructuring plan presented to the 11th Human Genome Mapping (HGM) Workshop, held in London last week, was met with vocal opposition from researchers.

This series of meetings has become a victim of its own success, as the assemblies have grown too large to stage effectively. But many researchers fear that the announced plans to limit attendance at future meetings to 150 participants would exclude all but HUGO officials and chairs of the increasingly autonomous chromosome mapping subcommittees, and the tide of feeling against the mapping workshop committee's plans ran high.

To be fair, HUGO President Sir Walter Bodmer emphasized that there was still a place for the large, old-style meetings, in addition to the chromosome subcommittees and the leaner, new-look plenary meetings, but plans for these are still vague and researchers are worried that the real business of genome mapping will be conducted elsewhere by a select band of HUGO apparatchiks, the big sessions

functioning essentially as window dressing. 'Grassroots' researchers, as one delegate put it, would feel excluded.

The problems are simply logistical. No fewer than 700 delegates squeezed into the elegant New Connaught Rooms conference centre in central London, and the locations of more than 600 new genes and more than 2,000 other DNA segments were announced. A densely printed 342-page book of abstracts is evidence enough for the intensity of the activity. The advances have been colossal: the first gene was mapped to an autosome in 1968, and fewer than 100 delegates discussed the mapping of about 60 genes at the first HGM workshop, at New Haven in 1973. Nowadays it is hard to find a venue that can cope with the demands imposed by hundreds of hungry, thirsty geneticists all at the same time.

For the moment, HGM 12 will go ahead as planned in Baltimore in 1992, and HGM 13 is pencilled in for Japan in 1993. But the style of these meetings is not entirely clear. Will they be hands-on business conferences or media circuses? The form they might take will, no doubt, be idiosyncratic and eclectic.

Henry Gee

Review panel blasts EPA report on EMFs

Washington

A BITING independent review has challenged much of a well-publicized report by the Environmental Protection Agency (EPA) on a possible connection between electromagnetic fields (EMFs) and cancer, and has recommended that the EPA rewrite the report almost completely.

When a draft version of the report was made public a year ago, it seemed to lend credence to theories that the EMFs surrounding power lines and electric machinery might promote cancer; an earlier, non-public draft had even suggested that EMFs be labelled as 'probable' human carcinogens. In response to the hoopla surrounding the report, the EPA asked that the Science Advisory Board, an independent body that advises the agency, assemble a committee to review the content and conclusions of the draft report.

The review panel, which included both EMF researchers and scientists from other, related fields, had little good to say about the EPA's report. It criticized everything from the executive summary ("Special care should be taken to insure that it should reflect the contents of the revised report exactly, without drawing any conclusions not substantiated by the body of the report") to the individual chapters ("The subcommittee finds that the report's discussion of epidemiologic findings is seriously deficient"), and concluded that "the draft report will have to be in effect rewritten."

The committee did not reject the possibility that EMFs could play a role in promoting human cancers, but rather concluded that the question of effects — harmful or benign — of EMFs on biological systems is "important and exceptionally challenging". The panel thought, however, that the EPA staff who produced the original draft report overstated and misinterpreted the available evidence, making the case for an EMF-cancer connection seem much stronger than it really is. (One small example, from among many: The chapter entitled "Supporting Evidence of Carcinogenicity" contains data that point toward biological effects of EMFs, but nothing that actually implies carcinogenicity, the committee noted.)

So it's back almost to the drawing board for the EPA. "We'll put together a plan for a new draft report over the next month," said William Farland, director of EPA's Office of Health and Environmental Assessment. The revised version will take into account the comments from the panel as well as from a second review with similar conclusions, and will add accounts of research done since 1989. The new draft will be ready in about a year, Farland predicted.

Robert Pool

MITI reveals 1992 budget

Tokyo

NANOTECHNOLOGY and brain-like computers are the new targets of research in the 1992 budget request of the powerful Ministry of International Trade and Industry (MITI). The ministry also plans to boost its already large budget for development of 'environment friendly' technology and to make a substantial budget allotment for reorganization of its research laboratories in Tsukuba, where much of the new nanotechnology research will be carried out.

The budget is scheduled to be submitted to the Ministry of Finance at the end of this month. The request is likely to undergo minor modification in negotiations with the ministry before it is submitted to the Diet for approval early next year, but science and technology budgets seldom experience major change during this process.

MITI's next-generation computer project to develop neural network and optical computers that mimic the human brain (see *Nature* 351, 336; 30 May 1991) is set to get underway next year with a budget of about ¥900 million (\$6.7 million). Ultimately, MITI hopes to pour several hundred million dollars into the project as the ministry has done for its predecessor, the fifth-generation computer project. In a surprising move, MITI has also made a substantial budget request (albeit reduced from this year) to continue development of the fifth-generation computer. The 10-year fifth-generation project, which sparked

competing government projects around the world, was due to end this fiscal year.

Another key budget item is a small request of ¥30 million (\$220,000) to begin a new large-scale (*ogata*) project to develop nanotechnology. MITI and industry hope to invest over \$200 million in the 10-year Angstrom Technology Project,

bon dioxide. The ministry requests ¥8,300 million (\$61 million) — 25 per cent above this year — largely for government/industry projects organized by the ministry's Research Institute of Innovative Technology for the Earth, which will open in 1992.

Under a painful process that has met much resistance, the ministry has been cutting back funding for development of coal-liquefaction technology because coal

liquefaction does not help reduce carbon dioxide emissions, and, thus, does nothing to alleviate current concerns about global warming. Instead, MITI plans to launch a new project next year with an initial budget request of ¥800 million to develop 'clean coal technology' that will reduce carbon dioxide emissions from coal use.

MITI and the Science and Technology Agency hope to increase funds for the Human Frontier Science Program, which supports international research on the brain and molecular biology, with a request for an additional 11 per cent for next year to support five more grants than the 30 budgeted for this year.

But it seems the programme has already reached close to the maximum ceiling the Ministry of Finance will allow.

The Intelligent Manufacturing System project to develop automated factories of the future, which is intended as a follow-up to the Human Frontier Science Program, also gets a significant boost to ¥810 million. But the hoped-for international cooperation in the project still remains in limbo, waiting for an agenda from the United States for an international feasibility study. **David Swinbanks**

MITI science and technology budget request	Thousand million yen	% change from 1991
Total R&D budget	264.5	+3.4
Japan Key Technology Centre	27.0*	-5.6
Basic technologies for future industries	8.3	+5.7
Large-scale industrial projects	15.0	+4.9
Sunshine new energy sources project	27.0	+8.7
Moonlight energy-saving technology project	11.9	+4.7
Unmanned space platform	5.8	+3.6
Fifth-generation computer	3.6	-49.7
Sixth-generation computer (NIPT)	0.9	+842.7
Global environment	8.3	+22.4
Intelligent Manufacturing System	0.8	+197.8
Human Frontier Science Program (HFSP)	4.2**	+10.7
Elucidation of biological functions (domestic HFSP)	0.3	-12.7
NEDO international grants	0.7	+53.4
Reorganization of Tsukuba science city institutes	14.9	+11.3

* Budget shared with Ministry of Posts and Telecommunications

** Budget shared with Science and Technology Agency

which will be centred at a new interdisciplinary research centre in Tsukuba science city (see *Nature* 352, 650; 22 August 1991). The ministry also allocates nearly ¥15,000 million (\$110 million) for the related reorganization of its institutes in Tsukuba.

MITI continues to pour more money into the development of technology to protect the global environment, such as CFC substitutes, biodegradable plastics, and technology to absorb and utilize car-

MITI hopes to foster outside interaction

Included in MITI's budget request (see above) are proposals to revise Japan's laws to encourage Japanese government laboratories and government-industry research consortia to interact more with the outside world.

One amendment will provide special tax exemptions for research consortia that include non-Japanese companies. The move is part of the ministry's policy of 'technoglobalism', which aims at encouraging interaction with overseas companies to counteract a perceived 'technonationalism' in the United States, where some government and industry officials are believed to be trying to bar Japanese access to leading US technology.

Under the proposed new law, which MITI officials expect to take effect next April, international consortia will be exempt from taxes that are normally imposed on any left-over funds consortia

have at the end of each fiscal year. At present, consortia have to spend all money in their fiscal-year account or face such taxation. This discourages them from carrying over funds to the next fiscal year.

A second amendment, proposed by MITI and the Science and Technology Agency, is intended to allow researchers in national laboratories to work for pay in outside organizations, such as private companies or overseas organizations, and to let government researchers receive funds from outside sources. At present, researchers in Japan's national research institutes are not allowed to receive outside remuneration or funds. This has led to the bizarre situation where national laboratory researchers cannot directly receive grants from the Human Frontier Science Program Organization in Strasbourg, France, even although Frontiers is funded largely by MITI and the Science and Technology Agency.

The proposed amendment will also allow Japanese government laboratories to offer larger salaries and longer-term contracts to non-Japanese scientists to try to attract foreign — and in particular Western — scientists to Japan's government institutes. Present laws require that non-Japanese government employees get the same low salaries as their Japanese colleagues but with none of the guarantees of long-term employment.

As another way of opening up MITI laboratories, the ministry has requested ¥1,800 million to provide 'academic discounts' to university researchers wishing to use facilities at MITI laboratories, such as the new microgravity centre in Hokkaido and the ion-engineering research institute in Kansai science city, where brand new high-tech research equipment is available for rent, at a price. **D.S.**

Australia boosts research

Sydney

As announced last week to the Federal Parliament, the Australian government will boost spending on science and technology by 4.3 per cent in real terms, to A\$2,600 million (\$2,050 million).

The budget, which now requires only routine approvals to pass through Parliament, includes a major boost for university research grants, while at the same time cutting funds for the Defence Science and Technology Organisation.

Funds for the centralized university research grants scheme, allocated by the Australian Research Council under a newly unified system, will be increased by 37 per cent, to A\$271 million (\$212 million). The extra money for the council should please researchers, who complained early this year that the council's inadequate funds made it too difficult to get grants for worthwhile projects.

The increase comes partly at the expense of university research and development funds allocated outside the grants system, which were cut by 6 per cent to A\$840 million (\$655 million). Still, the funds available for university research and development in 1992, counting both the central grants system and other funds, will be 6 per cent more than this year.

Australia's major research organization, the Commonwealth Scientific and Industrial Research Organisation (CSIRO), also did well, considering that the government has been deliberately squeezing the body's funds for several years. CSIRO's funding was increased by 3 per cent to A\$448 million (\$350 million), with the organization expected to get another A\$147 million from outside

sources such as licence fees and business support for projects.

The defense research organization was the major loser in the budget, as its funds will be cut by 6 per cent, to A\$221 million (\$173 million). No reason was given for the cut, and notes on the organization's research projects included in the budget papers did not say which programmes would be affected by the reduction.

The overall increase of 4.3 per cent in funding follows a 2.6 per cent rise last year and is in line with the government's emphasis on boosting research and development as a means of creating an advanced industrial base. Because Australia relies mainly on mining and farming for exports and has few of the very large high-tech companies found in other advanced countries, the government provides more than half the total funds spent on research and development.

Despite the increasing government emphasis on research, however, figures released with the budget show that private sector spending on research and development has increased very little. As a result, research and development incentives, including revenue lost through a tax concession, are expected to fall by 3 per cent to A\$362 million (\$282 million).

As another way to boost industry, the government this year began a cooperative research centres scheme, which is designed to create centres of expertise capable of passing on innovations to local industry. The first 15 centres were announced in March. More centres — eventually there will be 50 — are now being selected and will be revealed later this year.

Mark Lawson

AFRICA

University of Zimbabwe tumult

Cape Town

In protest at increasing government encroachment on academic freedoms at the University of Zimbabwe, vice-chancellor Walter Kamba announced his resignation at the university's mid-year graduation ceremony last month. Kamba's action was made more dramatic by the fact that the occasion was presided over by the progenitor of his discontent, Zimbabwean President Robert Mugabe, who was conferring degrees in his capacity as the university's chancellor.

The resignation, which has been followed by those of at least two other faculty members, is the culmination of a tumultuous semester at the university, in which students boycotted classes for three weeks in response to the Zimbabwe parliament passing the 1990 University

Amendment Act. This gives the government added representation on the university's council, and delegates authority to the vice-chancellor to hire and dismiss faculty and to expel students without any process of appeal — measures that Kamba was loath to implement.

Not everyone will be sorry to see the back of Kamba, whose record of administration during his ten-year tenure has by all accounts not been exemplary. But many academics are even more apprehensive about who will replace him when he leaves office at the end of next month. There are fears that the government will appoint a hardliner from outside the academic sector to 'sort out' activists among the 10,000-strong student body who are proving to be a thorn in the side of the ruling party.

Michael Cherry

Fullerenes warm up

Tokyo

THE temperature at which doped fullerenes can become superconducting seems to be on a dramatic rise. Last week, a group of Japanese researchers claimed they have produced an iodine-doped fullerene that superconducts at 57 K — 15 K warmer than the previous record set only a few weeks ago by a team at Allied Signal's laboratories in the United States (*Nature* 352, 463; 8 August 1991). Allied Signal's record surpassed one of 33 K set by researchers at Japan's NEC company in July (*Nature* 352, 222; 18 July 1991).

The Japanese group, led by Hisashi Sekine of the National Research Institute for Metals in Tsukuba science city, says two papers on their research on halogen-doped fullerenes have been accepted for publication in the 1 October and 15 October issues of the *Japanese Journal of Applied Physics*.

David Swinbanks

BRAZIL

Science minister takes on a pineapple

São Paulo

Physicist José Goldemberg has been transferred from his position as Secretary of Science and Technology to take over Brazil's troubled education ministry. Edson Machado, Goldemberg's number two man at the science and technology department, has been given the top job there, but the possibility remains that president Fernando Collor de Mello may decide to merge the two units, creating a 'super ministry' of science, technology and education.

As education minister, Goldemberg's most immediate concern is the state of the federal universities, which consist of about 30 units around the country. The staffs of those universities have been on strike demanding better wages for more than two months.

Goldemberg has criticized the federal institutions, where — with the exception of a few departments — tenure is easy to obtain and little research is done. He has said he will try to create some concrete way to evaluate work at the universities.

In the past 20 years, Brazil has had 11 ministers of education, all but two of whom were politicians or military officers with little or no experience with teaching or education. Goldemberg told an audience of scientists a few weeks ago that whenever Collor tried to speak with him about the Ministry of Education, he would attempt to change the subject. He said he considered the job to be an "abacaxi" — Brazilian slang for an unwanted and bothersome problem, literally a "pineapple". Now Goldemberg's friends fear that this is one fruit that may prove rather hard to digest.

Ricardo Bonalume

China passes copyright law

Hong Kong

CHINA, long regarded as a black hole for protection of intellectual property rights and copyrights, has begun to implement its first copyright law. This could prove to be the initial step in opening China up to a variety of imports, particularly computer software, but critics say the country still has a long way to go before innovators can trust that their work will not be used without credit or compensation there.

The new copyright law went into effect at the beginning of June, and a supplementary law that deals with software regulations will take effect 1 October. The legislation does not specifically deal with patents, which are covered by an existing statute that Western countries have complained is relatively lax.

Still, the copyright law should cause a major improvement in opportunities for doing business in China. According to the State Copyright Bureau, which has the responsibility for implementation, the law will — at least to some extent — help reduce the violations of copyrights, patents and intellectual property that have made Western companies wary of operating in China.

Passage of the law follows several years of complaints by European and US companies that their copyrights and patents gave them essentially no protection against unauthorized copying in China. Industry sources estimate that pirating of copyrighted goods in China costs the United States alone about \$430 million each year. The Business Software Alliance, a Washington-based group that lobbies for stronger protection of intellectual property

rights worldwide, refuses even to estimate the total of indirect losses that the software industry has suffered because of the lack of protection. In the absence of a copyright law, it claims, companies have decided not to sell in China, thereby missing out on huge but uncountable profits.

The Chinese government has gone some distance to meet foreign complaints in the language of the new law. For example, it permits companies to own the copyrights of published works created by their employees. An early version of the legislation would have given the copyrights to the employees, and would have permitted their companies to use the work without permission for only two years. In addition, the law limits the use of copyrighted works, such as software, by education institutions. Initially, educational establishments would have had virtually unlimited use of copyrighted material, setting up the possibility that China could copy and distribute specific pieces of software throughout its vast educational system without paying royalties.

No one expects the new copyright law to be a panacea, however. For example, the State Copyright Bureau has recently embarked on the task of publicizing the legislation and educating people on intellectual property and related matters — and has found it a tough challenge, according to Shen Rengan, Copyright Department Director of the Bureau. "It takes time for people on the mainland to get familiar with the law, as they have long been weak in the legal sense of identifying intellectual property and physical property, thus posing difficulties to the Bureau when putting the law into effect," Shen told a recent seminar in Hong Kong.

Groups such as the Business Software Alliance complain that the legislation is too weak. One major objection is that neither the law nor the regulations protect works of foreign authors published abroad in the absence of a bilateral or multilateral treaty to which China and the author's country are parties. At present, China is party to no such treaties. However, Shen said China is negotiating with the government of Hong Kong and local authors' associations on possible pacts.

The software alliance also complains that computer programs are not protected as literary works, and that neither the law nor the regulations provide adequate provisions for enforcement. Some observers have argued that the law went into effect at a convenient time to help China's cause in the struggle in Washington over achieving Most Favored Nation status — and that the law is designed to protect China from US trade sanctions more than Western companies from Chinese counterfeiters.

Peter Gwynne

US Greens go national

Washington

THIS week sees the launch of a new political party in the United States. The US Green movement — until now a loose coalition of local organizations — is forming the Green Party-USA, a national body headed by a seven member coordinating committee. The launch of the US party comes as support for the Greens in Europe seems to be waning, now that many of the major European political parties have repackaged their own policies to stress environmental measures. But Charles Betz, a member of the national coordinating committee, is undeterred by these developments. "If you're a Green, you're in it for the long haul," he says.

Just how a national US Green party will function is still unclear. Betz says that the party will not lobby on Capitol Hill, and that even a narrowly defined set of policy goals is unlikely: the 'grassroots' nature of US environmental activism prevents a central committee from dictating policies to local Green organizations, he says. Instead, the committee will encourage local groups to participate in broadly defined national campaigns, starting with a drive to promote the use of solar-generated electricity. The US Greens' electoral ambitions will for the time being be limited to municipal, and perhaps state elections.

Peter Aldhous

SPACE MISSIONS

Galileo rescue fails

Washington

THE latest attempt to open the jammed main antenna on the National Aeronautics and Space Administration's (NASA) Galileo Jupiter probe has failed. Officials at the Jet Propulsion Laboratory in California had hoped that turning the spacecraft for 50 hours to shield the antenna from the Sun would release several of the ribs supporting the umbrella-like structure that are apparently stuck against the antenna's central tower (see *Nature* 352, 655; 22 August 1991). But the cooling did not free the carbon fibre ribs, thought to have become jammed against a series of metal pins on the central tower.

Mission scientists will now turn their thoughts away from the troublesome antenna, to concentrate on Galileo's encounter with the asteroid Gaspia, on 29 October. Data from this flypast will be stored on an on-board tape recorder, and will be transmitted back to Earth later, using the craft's back-up antenna — or the main antenna, if it opens. To return most of the data from Galileo's 1995 rendezvous with Jupiter and its moons, however, a functioning main antenna is essential. NASA plans another cooling manoeuvre in December, when Galileo is farther from the Sun.

Peter Aldhous

British Science

WE shall be publishing on 12 September *Nature's* Manifesto for British Science, which will be offered without charge to any one of the political parties contesting the next general election, and in the belief that it is an improvement on their own version.

Following publication, on Friday 13 September, there will be a public meeting at the Royal Institution, Albemarle Street, London W1 at which Sir Mark Richmond, chairman of the Science & Engineering Research Council, will be the principal speaker. The chairman will be John Maddox, Editor of *Nature*. The meeting will start at 9.45 a.m. and end at 12.45 p.m. The political parties will be invited to send representatives, but there will be ample time for discussion. Those wishing to attend should apply for tickets to Mary Sheehan, *Nature*, 4 Little Essex Street, London WC2R 3LF.

Publicity on controversial data

SIR — In the light of the enormous publicity that followed the publication by Jacques Benveniste in *Nature* in June 1988 (ref.1), it is highly remarkable that a letter which appeared in *Nature* on 23 November 1989 (ref.2) remained apparently almost unnoticed. In this letter, the author describes results of a test by an unidentified "eminent homoeopathic practitioner, a former President of the Faculty of Homoeopathy" to select from 20 bottles containing either "natrium muriaticum 30°C" or "sulphur 30°C". The investigator was told that the contents of the bottles had "strikingly different properties" and "were strongly active". The bottles were blinded. The investigator was allowed to use any method for identifying any "distinction".

The outcome of the test was as might be expected: no distinctions were found, the results obtained "might just as well have been arrived at by chance alone".

Not only does the letter of 1989 not mention the name of the person who carried out the homoeopathic test, it does not give any information either on the nature of the tests which have been carried out, which seems to be unique for *Nature*. After some enquiries, I was informed about the actual test: the so-

called emanometer had been applied. In a very diffuse way, this 'instrument' has been described by Ritchie McCrae and F.F. Hom³. In this paper the impression is given that with the emanometer the efficacy of homoeopathic products can be proved. The outcome of the study in 1989 is absolutely negative, but the author of the letter is still not convinced of the inactivity of the 30°C dilutions. He suggests that a clinical trial should be carried out: the circle is round indeed.

A few years after the 'Benveniste story', Benveniste himself announced: "... I will (try to) publish in the months to come indisputable proof"⁴. So far I have not seen such a paper. Scientific journals should stop publishing papers that deal with obscure techniques or nonsense theories, as it is very likely that they will be misused⁵.

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Lesson for science

SIR — With the publication of the draft report by the NIH Office of Scientific Integrity¹, the most important lessons for science in the Baltimore controversy threaten to become lost in the ensuing squabbling. These lessons are twofold.

The first is that, protestations to the contrary notwithstanding, the scientific endeavour tends to resemble Kuhn's description of science as a triumph of expectations over reality². Initially conceived test-hypotheses solidify as dogmatically held paradigms, impervious to refutation. In this instance, David Baltimore states his reliance on the authority of his co-author ("Imanishi-Kari provided the expertise in serology that I lacked"³) over Margot O'Toole's experiments indicating non-replicability of a co-author's conclusion. If the most renowned of the co-authors cannot understand the basis on which his own paper's conclusions rest, what must that imply for those of us who are not Nobel laureates? So much for the vaunted "rigours of the scientific peer review process" to uncover error. Or is O'Toole not a peer?

Second, and more important, is the threat to the vigour of scientific debate. One of our leading scientific institutions, whose adherence to experimental inquiry led Oswald Avery and colleagues to the trail of DNA as the inheritance

factor at the then Rockefeller Institute, a decade before the discoveries of Watson and Crick^{4,5}, may now be headed into an era based on hypothesis as unrefutable dogma.

Should it not now be our concern to focus on the central problems this controversy raises? Why did not O'Toole's efforts find a publication outlet? Why could not a 1987 paper by Walter Stewart and Ned Feder outlining discrepancies in the original *Cell* paper also find a scientific journal willing to publish their doubts⁶ [see footnote]? As James Lovelock observed in these pages⁷, the evolution of scientists into dogmatic authoritarians poses the danger of an acceptance of a censorship of ideas. As this case reveals, the danger is also to the integrity of the process of scientific inquiry itself, the censorship of validity checking. That should be our central concern in this matter.

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New words

SIR — Friedrich Katscher complains (*Nature* **351**, 179; 1991), and quite rightly too, that scientists who do not grasp the intricacies of Greek and Latin grammar should be more hesitant about creating modern words in which the mysteries of declension render the scientific meaning incoherent to anyone unfortunate enough to be conversant with these two languages.

A 'mutant' (properly signifying 'that which is in the process of mutating') is clearly a solecism, and if Katscher thinks 'mutatus' too ugly, may I suggest a 'mutate' in the hope that scientists will still be able to tell the difference between the word's use as a noun and a verb?

And can anyone enlighten me on how physicists came to imagine that 'accelerate' denotes 'any change in velocity or direction', when even a passing acquaintance with Latin (or English) would seem to indicate otherwise?

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SIR — Friedrich Katscher's suggested term 'recombined' is undesirable as an alternative to 'recombinant'. Participial adjectives are formed directly from participles, and the suffix *-ated* is not regularly present where the verbal stem does not itself contain *-at-*. The form 'recombined' has apparently been generated as a back-formation from 'recombination', but it is in effect a formal derivative of a non-existent verb **recombine*. (Compare, for example, the regular noun 'computation' and the unacceptably irregular **computed*).

Although there is no objection to the regular form 'recombined', there is another perfectly possible adjective of past participial form; *recombinate*. This represents the common English development from the Latin suffix *-atus*, and has the added advantage of readily forming a corresponding noun, also spelt *recombinate*.

The analogous adjective and noun 'mutate' (stress on the first syllable) is used in certain restricted contexts, but is awkwardly confusable with the verb; however, Latinate forms such as 'mutatus' are not easily assimilated into natural English use, and are perhaps best avoided. I think 'mutant' is philologically defensible: there are precedents for words ending in *-ant* which have lost a present participial sense, though they are few (*convenant*, *descendant*, *immigrant*, and *quadrant*, for example).

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New technologies needed

SIR — I am appalled by your article "Many catastrophes too many" (*Nature* 351, 171–172; 1991), and its reaffirmation of simplistic and fatally incorrect mainstream ideas on the economic development of poor nations. Modern economic theory, which is founded on the notion of continual growth, largely ignores ecological principles. Because the Earth, and all the renewable and non-renewable resources it contains, is finite, economies, and the populations they support, cannot grow indefinitely.

The unprecedented increase in world population this century has been at the expense of a staggeringly high rate of depletion (on a geological time-scale) of resources: fertile soils, timber, freshwater aquifers, mineral and fossil fuel deposits. These resources, built up over periods ranging from centuries to millions of years, are being used up in mere decades. As resources are depleted, the rate of development of all nations, industrial as well as rural, will begin first to slow and then to shrink. Economists suggest that new resources will be discovered to replace declining ones. This may be true in isolated instances (plastics replacing metals), or for a limited time (fossil fuels replacing biomass fuels). However, the present scale of loss of soil, water and fuel resources is so great that the only credible substitution would be an entire new planet.

In the cold light of ecological reality, the natural resources necessary for the industrialization of poor countries do not exist now, and will not in the foreseeable future.

In 1988, the combined global gross national product (GNP) was \$15 million million for 5,000 million people, an average per capita income of \$3,000, which supports the typical lifestyle of citizens of Portugal or Venezuela. But the average per capita income of the 1,000 million people in the most industrialized nations was \$11,275. To raise the income and lifestyle of the rest of the world to this level, the total GNP would have to increase almost fourfold, to more than \$50 million million, even if the human population does not increase further. Because of the present pyramidal age-class demographics of the world population (more children than adults), even if a two-child per family limit were imposed in every country at this instant, the number of humans would still nearly double before levelling off. With less drastic population control measures, the projected peak in human population will be more than 15,000 million by the end of the next century.

Given that the global GNP is unlikely to increase much further (a decrease is

more probable due to resource depletion), the implication is that, in the future, average per capita income will decrease to a half or even a third of the present value. Rather than the industrial development and demographic transition now expected for rural countries, a decline of all economies is more probable. If present inequities in income distribution in the world do not change, then, while the citizens of industrialized nations will merely suffer reduced circumstances, the poor are likely to fall off the edge of existence.

It is popular to deride ecological Cassandra because the Green Revolution of the 1970s temporarily matched food production and the demands of population growth, but one should not forget that in the legend the prophetess foretold correctly. Another agricultural great leap forward is not on the cards. We can no longer afford to blind ourselves to the true nature of our global predicament, which includes a changing climate due to global warming as well as a declining resource base. Appropriate new technologies based on sustainable resources, as well as population control policies, will be required for all nations, not only poor ones. It is not too late to confront reality. If we do not, then global civilization itself is at risk of disintegration.

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SIR — The leading article "Many catastrophes too many"¹ suggested approaches to disaster relief and long-term assistance for developing countries, but how would we react to devastation in the industrialized world? If we have to rebuild Los Angeles after the 'big one' hits Southern California, who would support the reconstruction of the notorious freeways and parking lots catering to photosynthetic smog produced by internal combustion engines? If we had to recreate the British power grid, would we plan generating stations requiring a Windscale/Sellafield?

Developing countries are faced with the choice: they can emulate the industrialized world and knowingly make ecological 'messes' (as suggested in the leading article) in exchange for reliance on largely imported fossil fuels and nuclear energy, or they can — ideally with North-South collaboration and assistance — follow a route towards greater self-sufficiency and sustainable development using indigenous renewable energy resources.

Your leader writer is unfortunately not alone in rejecting renewable energy systems as a phenomenon of 'Europe's dark ages' but to do so is to confound appropriate technology with primitive technology. For instance, 20 years of innovation have made windmills a viable alternative with the potential to supply 10 per cent of Britain's energy needs². Biomass and solar energy systems are now highly efficient, capable of producing electricity for \$0.04 per kilowatt-hour³.

Through a process that emphasizes collaboration rather than simply the donation of technology, solar ovens and other wood-saving systems have been successfully integrated into rural villages in Central America⁴. These advances are particularly striking in the light of the small fraction of energy research funding allocated for renewables, the lack of support for efforts to develop technologies suited to the needs of developing nations⁵ and the range of economic impediments faced by companies developing renewable energy systems⁶.

Despite the immense potential of solar and windpower, the established paradigm sees renewables as having only a minor role in the economies of developed and developing countries alike. This outmoded point of view results in misinterpretation of data on modernized renewable energy technologies. Terence Price's recent Commentary⁷ exemplifies this line of thought: the possibility of a large-scale contribution from renewables is dismissed while *rapprochement* with nuclear power is advised.

A paradigm shift is in order. The obvious environmental advantages of renewable energy systems do not have to be bought at the price of economic development (although renewable energy alone will not save us from the ecological effects of over-consumption and resource exploitation). The integration of environment-friendly appropriate technology into international development assistance is not a luxury, it is a necessity.

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US government orders new look at dioxin

The Environmental Protection Agency is evaluating data from the past decade that suggest dioxin's toxicity may be overestimated. A risk assessment model based on biological mechanism is being drawn up.

DIOXIN is widely thought of in the United States as the most toxic chemical known to man. As a component of the infamous Agent Orange that the US used to defoliate the jungles of Vietnam, after the war dioxin was blamed for cancer in servicemen who saw duty there. Although compensation was finally offered, neither solid epidemiological nor biological proof of cause and effect has been conclusively demonstrated.

When a chemical plant exploded at Seveso, Italy in 1976, spewing dioxin-contaminated agents into the air, it was feared (and assumed) that the exposed citizens of the town would experience increased cases of cancer and/or birth defects. In fact, severe chloracne was reported but, fortunately, more life-threatening diseases were not.

When, in 1982, the soil in the small town of Times Beach, Missouri was found to be contaminated by dioxin at levels around 1 part per million, US government officials closed the town down permanently and evacuated its 2,000 residents to safer ground across the river. Nearly a decade later, clean-up work is still going on. Someday, when the soil is incinerated and the buildings are all torn down, Times Beach may be turned into a park.

Over the years, hundreds of millions of dollars have been spent getting rid of dioxin, which has earned its reputation as a killer because it is highly carcinogenic in guinea pigs and causes birth defects in mice exposed to small concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD.

Now, into this politically charged arena comes William K. Reilly, the head of the US Environmental Protection Agency, saying that dioxin may not be so exceptionally toxic after all. During the past several years, data in humans has accumulated that question the validity of extrapolating TCDD data from guinea pigs to man. Reilly has called for a total re-evaluation of dioxin in all of its many chemical forms (there are 75 or so).

It takes a brave man to put scientific evidence ahead of deeply ingrained public opinion. The last time a federal official suggested that dioxin standards could be relaxed was in 1989 when Vernon Houck of the Centers for Disease Control in Atlanta advised the state of Georgia about water standards. Shortly thereafter, he was subjected to a congressional inquiry into his possible bias toward the paper indus-

try which puts dioxin-contaminated wastes into streams after bleaching pulp. Houck stood his ground, testifying that "new information indicates that we should be less concerned than we once were."

In March of this year, Reilly and other top EPA officials received a briefing on dioxin that Reilly credits for his change of mind. First, his advisers reported an unusual consensus from a recent dioxin conference at the Banbury Center at Cold Spring Harbor, New York. Researchers agreed that the toxicity of TCDD depends upon its binding with the aromatic hydrocarbon receptor, or Ah. Most to the point, they agreed that receptor binding mediates TCDD toxicity in virtually every test system studied, and that receptor binding needs to occur in thousands of cells, though the dividing line between safe and toxic concentrations is not known.

Nevertheless, this observation leads directly to Reilly's conclusion that "work should begin on a new biologically-based model for assessing the toxicity of dioxin." Until now, the Environmental Protection Agency has relied on the standard linear multistage model that predicts no threshold of safety. Based on this model, US standards for exposure to 'background' levels of dioxin that is in the environment are stringent: .006 picograms per kg body weight per day for intake from food or water, for instance. Interestingly, Canadian and European scientists generally have not accepted the US position that dioxin is the most toxic chemical around. Conservative estimates put a safe dioxin intake at 1 picogram per kg per day, while more liberal standards go as high as 10 picograms per kg. Although no changes in US regulations have been put forward, it is likely that the US will move in the direction of its neighbours abroad.

A second bit of data that figured in Reilly's request for a new look at dioxin comes from a large epidemiological study reported in the 24 January issue of *The New England Journal of Medicine*. Researchers at the US National Institute for Occupational Safety and Health in Cincinnati, Ohio, conducted a retrospective mortality study of 5,172 workers at 12 chemical plants that produce agents in which TCDD is a contaminant. Their conclusions: "Mortality from several cancers previously associated with TCDD (stomach, liver, and nasal cancers, Hodgkin's disease and non-Hodgkin's lymphoma)

was not significantly elevated in this cohort. Mortality from soft-tissue sarcoma was increased but not significantly."

Government scientists, working with colleagues in universities, have been asked not only to develop a biologically based or receptor model, but also to construct a plan for evaluating the new epidemiological data so that any new standard-setting regulations encompass the new data as a whole. March of 1992 is the target date for completion of the work.

Environmental Protection Agency scientists seem to be fully aware that they are entering dangerous territory. Even though data may exonerate dioxin somewhat, it remains a toxic contaminant of the environment that has no compensating benefit. Furthermore, a reassessment of dioxin has obvious implications for related polychlorinated biphenyls. Ultimately, it could lead to a biologically based model for any agent or group of agents for which toxicity is understood at a mechanistic level.

That the government must proceed carefully, and openly, is vital. Reilly has promised independent scientific review, which is certain to be subject to harsh scrutiny by groups committed to the view that the only acceptable risk is no risk at all. Over the years, this has become the unstated goal of many groups in the United States, but with science's ability to measure chemicals at ever smaller concentrations, it becomes crucial to rank toxic compounds in some sensible order of hazard. As Reilly has noted, "There simply are more anxieties than we can possibly create laws to alleviate, and far more risks than resources to eliminate them."

There is no doubt that a decision to declare dioxin less hazardous will be painful, particularly to the people at Times Beach and elsewhere whose lives have been so totally disrupted. At the time steps were taken to evacuate the town, the decision was entirely in keeping with scientific data available. Human data were scarce. It was thought prudent to rely on animal data and they made a strong case for judging dioxin to be unusually toxic. Although researchers may be comfortable with the idea that new judgements should correctly follow new data, it remains a difficult concept for the public.

Nevertheless, it is time to set priorities, even if it is politically treacherous.

Barbara J. Culliton

Immunosuppressants at work

Anthony L. DeFranco

To the transplant surgeon, the powerful immunosuppressants cyclosporin A and its new cousin FK506 qualify as wonder drugs. To the cell biologist and immunologist, the mechanism of action of these drugs constitutes a challenging puzzle. Reports by Flanagan *et al.* on page 803 of this issue¹, and by Liu *et al.* in last week's *Cell*², now add two pieces to the puzzle by tying the action of the drugs to calcium signalling elements and transcription factors.

Early immunological studies revealed that cyclosporin A (CsA) blocks activation of T cells and that this is partly the result of inhibition of synthesis of interleukin 2 (refs 3, 4), the main growth factor for T cells. As the logical descendant of this experimental approach, the work of Flanagan *et al.*¹ reveals the main molecular site of this block.

The gene for interleukin-2 (IL-2) is silent in resting T cells, but is activated upon antigen recognition and signal transduction by the T-cell receptor complex. Cyclosporin A and FK506 block this antigen-induced transcription of the IL-2 gene. Several of the transacting factors that bind to the transcriptional regulatory region of the IL-2 gene are affected by CsA and FK506, including

AP-3, NF- κ B and, perhaps most dramatically, NF-AT (nuclear factor of activated T cells). NF-AT is undetectable and its corresponding binding site is transcriptionally inactive in all cell types other than activated T cells. The T-cell antigen receptor sends its signals by tyrosine phosphorylation and phosphoinositide breakdown⁵. It seems to be the second event that induces the appearance of NF-AT, as NF-AT is induced fully by the treatment of T cells with the combination of phorbol esters, which mimic diacylglycerol in activating protein kinase C, and a calcium ionophore, which bypasses inositol trisphosphate in elevating intracellular free calcium. If NF-AT is in some way especially dependent upon the intracellular step(s) blocked by CsA and FK506, its restricted distribution might explain the selective inhibition of T-cell activation by these agents.

What, then, is it about the induction of NF-AT that is blocked by the immunosuppressive agents? Flanagan *et al.*¹ have discovered that the induction of NF-AT is unique among transcription factors. The molecule is made of two components. One is induced by phorbol ester treatment, is located in the nucleus and requires new protein synthesis. The other is pre-existing and cytosolic, and following an increase in levels of calcium translocates to the nucleus to combine with the first component. Cyclosporin A and FK506 block the translocation from the cytoplasm to the nucleus of this second entity, but not the appearance of the induced nuclear component (Fig. 1). These results are in agreement with a large amount of work that shows that it is a calcium-dependent step that is blocked by the immunosuppressive agents⁶⁻⁸.

The second new insight² comes from a biochemical approach: find what these agents bind to in the cell and perhaps that will indicate what process they interrupt. Indeed, CsA binds with high affinity to an abundant cellular protein, dubbed cyclophilin (now cyclophilin A). Cyclophilin A was purified, the gene encoding it was cloned, and the sequence was found to match the

sequence of an enzyme, peptidyl-prolyl *cis-trans* isomerase. This enzyme speeds up the isomerization of proline peptide bonds in polypeptides, and thereby facilitates protein folding. Importantly, binding of CsA to cyclophilin blocked the isomerase activity. Thus, the straightforward model arose that CsA works by inhibiting the normal cellular function of the peptidyl-prolyl isomerase.

This hypothesis received a big boost from studies with FK506, a structurally distinct molecule that blocks T-cell activation in the same way as CsA. FK506 does not bind to cyclophilin A, but it does bind to another abundant, ubiquitously expressed protein, called FK506-binding protein (FKBP or FKBP12). This protein was also purified and cloned, and was found to bear no obvious sequence similarity to cyclophilin A, a conclusion now supported by comparison of the three-dimensional structures^{9,10}. Amazingly, FKBP is also a peptidyl-prolyl isomerase. With this revelation, it becomes difficult to argue that these prominent binding proteins ('immunophilins') and their enzymatic activities are irrelevant to the actions of the immunosuppressants. Moreover, a gene in *Drosophila*, *ninaA*, which was identified as being necessary for the operation of the visual system, has strong sequence similarity to cyclophilin A. Although peptidyl-prolyl isomerase activity has not yet been demonstrated for the *ninaA* gene product, it does appear to act on insect rhodopsin post-translationally to allow proper expression¹¹, a result that is consistent with a role for cyclophilins in protein folding.

Nonetheless, there are problems with the hypothesis that CsA and FK506 act by binding to the immunophilins and blocking their activity. Why are CsA and FK506 strongly inhibitory for T-cell activation, but apparently not involved in the functions of so many other cell types in which immunophilins are expressed? If the immunosuppressants act by inhibiting the corresponding binding proteins, why will either agent block IL-2 production completely, when it binds to and inhibits only one of the two peptidyl-prolyl isomerases? Worse yet, the concentrations of immunosuppressants needed for inhibition of T-cell activation are well below the concentrations needed to saturate binding to the corresponding immunophilin⁴. Finally, there is doubt as to whether the peptidyl-prolyl isomerases would speed up folding by more than several fold⁹; could inhibition of such a process explain the dramatic suppression of IL-2 production?

Although none of these questions hanging over the hypothesis was deci-

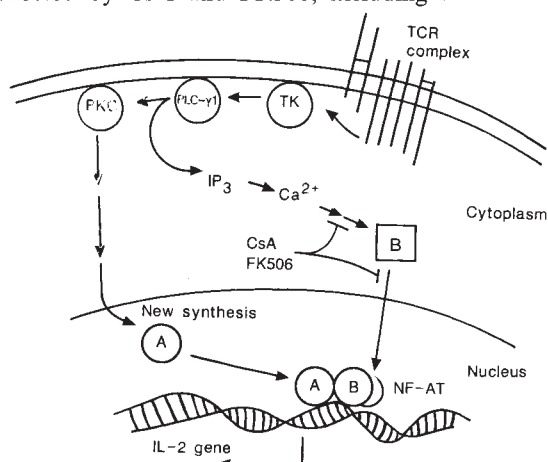


FIG. 1 T-cell signalling induces the appearance and activity of nuclear factor of activated cells (NF-AT). Two components of NF-AT combine to create the DNA-binding activity. The simplest hypothesis is that NF-AT is a DNA-binding protein that has two subunits, both of which are required for DNA binding, with one subunit being the pre-existing cytosolic component and the other being the induced nuclear component. Cyclosporin A (CsA) and FK506 block the translocation of the cytosolic component to the nucleus. It is not yet known whether these drugs block the action of a calcium-signalling pathway on the NF-AT component, or whether they block the ability of the component to translocate following action of the calcium signalling pathway, so arrows are shown for both of the possible steps at which these agents may act. TCR, T-cell antigen receptor; TK, protein tyrosine kinase; PLC- γ 1, phospholipase C- γ 1; PKC, protein kinase C; A and B, the nuclear and cytosolic components of NF-AT, respectively.

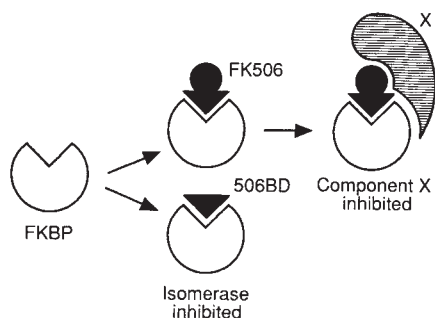


FIG. 2 Model for the mechanism of immunosuppressant action. Both FK506, which is an immunosuppressant, and the analogue 506BD, which is an antagonist of immunosuppression by FK506, bind to FKBP and inhibit its peptidyl-prolyl isomerase activity. From several lines of evidence it seems that inhibition of the isomerase activity cannot account for immunosuppression. Rather, it appears that the FK506-FKBP complex, but not the 506BD-FKBP complex, can interact with another component (X) in the cell and suppress IL-2 transcription and T-cell activation through this component.

sive, clearly what was needed was an *in vivo* test of the biological importance of the inhibition of peptidyl-prolyl isomerase activity. Such tests have come from two directions, and they both cast considerable doubt on the idea that inhibition of peptidyl-prolyl isomerase can account for the action of the immunosuppressants.

One approach was to take advantage of the ubiquity of the immunophilins. The yeast *Saccharomyces cerevisiae* expresses highly conserved versions of both cyclophilin A and FKBP. Interestingly, both CsA and FK506 block the growth of the yeast cells. Resistant mutants can be isolated that completely lack the corresponding binding protein^{12,13}. This result shows that the immunophilins are essential for the ability of the drugs to act, at least in yeast. Moreover, loss of either immunophilin is not lethal. Therefore, inhibition of its activity would not be lethal either, and the ability of the drug to inhibit cell growth must involve binding to the corresponding immunophilin, but does not depend solely on inhibition of isomerase activity. A similar conclusion was derived from studies of chemically modified versions of FK506. One derivative, called 506BD, inhibits FKBP's isomerase activity, but does not inhibit production of IL-2. In the T cells, 506BD does bind to FKBP at the same site as FK506, as it will block FK506's action in a competitive way. A second derivative called rapamycin behaves similarly in this regard⁴. Taken together, these results lead to a different model, in which CsA or FK506 binds to the corresponding immunophilin and the resulting complex actively inhibits some cellular process⁴ (Fig. 2).

This alternative hypothesis for the mechanism of immunosuppressant action

suggests that the drug-immunophilin complex should bind to a cellular target protein. To test this idea, Liu *et al.*² used the immunophilins to make affinity columns. Both the complex of CsA and cyclophilin and the complex of FK506 and FKBP, but none of the individual components, bind specifically to three polypeptides from bovine brain, thymus or spleen extracts: calmodulin and the two subunits of calcineurin, a calcium-activated serine-threonine protein phosphatase. In both cases, the interaction of the immunophilin seems to be with calcineurin; calmodulin can bind to calcineurin independently. Thus calcineurin could be the target of the drug-immunophilin complexes. In accord with this possibility, the drug-immunophilin complexes block the calcium-activated protein phosphatase activity of calcineurin. The specificity of these interactions fits the specificity of inhibition of IL-2 production: the complexes of rapamycin or 506BD with FK506 fail to interact with or inhibit the activity of calcineurin.

So the new results provide strong support for the alternative model that the immunosuppressants work by binding to immunophilins and then inhibiting some other component (Fig. 2). More-

GALACTIC MAGNETIC FIELDS

Spinning a tangled web

Ellen Zweibel

SPIRAL galaxies, including our own Milky Way galaxy, are permeated by magnetic fields which significantly affect the dynamics and energy balance of the interstellar medium. The fields' influence is important both in small-scale processes such as star formation and in the global equilibrium of the interstellar gas. The relativistic-particle, or cosmic-ray component, which heats and ionizes the interstellar medium as well as contributing significant pressure, is confined by the magnetic field and probably accelerated by hydromagnetic processes. Observations of the barred spiral galaxy M83, described by Neininger *et al.* on page 781 of this issue¹, provide a beautiful example of just how complex the gas-field interaction can be.

The local properties of the magnetic field in our Galaxy are quite well known, but the global morphology is uncertain. Therefore, the ability to map magnetic fields in other spiral galaxies provides an alternative way to identify the large-scale characteristics. It may even shed light on the origin and evolution of galactic magnetic fields. Only recently has such mapping become feasible.

One way of observing the fields is through the nonthermal radiation spiral

over, it is striking that the drug-immunophilin complexes block a calcium-induced translocation of a component of NF-AT and that they inhibit the calcium-activated protein phosphatase calcineurin. Could it be that CsA and FK506 block a dephosphorylation of the cytosolic component of NF-AT, and that this dephosphorylation is required for transit to the nucleus? It looks as if the pieces of this fascinating puzzle are starting to fit together. □

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galaxies emit at radio frequencies. This signal is almost certainly synchrotron emission radiated by cosmic-ray electrons as they spiral through the galactic magnetic field. The intensity is proportional to the magnetic energy density (or square of the magnetic field strength). If the field direction is uniform, the radiation will be strongly linearly polarized perpendicular to the projection of the field on the plane of the sky. Fluctuations in the field direction on angular scales smaller than the telescope beam reduce the polarization below its theoretical maximum. Therefore, the degree of polarization furnishes information about the relative strength of fluctuations of the field.

M83, the galaxy discussed by Neininger *et al.* in this issue¹, has a well-organized spiral structure, probably driven by its strong central bar (see figure). It is a starburst galaxy², which is to say that massive stars are being formed in it at an unusually high rate. The spectrum of its X-ray emission suggests that it has a hot, gaseous halo³.

The new observations show that the magnetic field direction faithfully traces the optical spiral structure and is also well aligned with the central bar, as

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Although a spiral galaxy like our own Milky Way, the starburst galaxy M83 differs in that it has a strong bar of stars across its centre (lying horizontally in this image). It is also notable for the high frequency of supernova explosions — one every 10–15 years — exceeding that of any known galaxy.

noted previously⁴. The degree of linear polarization is greatest in the spiral arms and is reduced by a factor of 2–3 in the inner regions. Sukumar and Allen⁵ reported reduction by an even larger factor (but the effect is clear even if the magnitude is uncertain). Their maps, made over a larger spatial area, show well-ordered magnetic fields well beyond the optically visible gaseous disk.

The magnetic field strength in the galaxy averaged over the disk is estimated to be about 11 microgauss, with the strength of the ordered component being around 4 microgauss and the random component around 10 microgauss. These values are between two and three times the strength of the local magnetic field in our Galaxy⁶, which has a similar ratio between the ordered and small-scale fields.

Information about the full three-dimensional structure of the field can be derived from observations of Faraday rotation; this is a rotation of the orientation of the polarization vector produced when there is a component of magnetic field along the line of sight. Analysis of the Faraday rotation measure across M83 shows that there may be a significant component of well-ordered field perpendicular to the galactic plane.

What can be made of these observations? The first interesting point is the increased disorder of the field close to the galactic centre. The rotation of interstellar gas about the centre of M83 is significantly perturbed by the stellar bar, which rotates more slowly than the gas. The gravitational forces associated with the bar lead to standing shocks in the gas and to amplification of the component of

the magnetic field tangent to the bar⁴. This effect also operates along the spiral arms, and is probably responsible for the close correspondence between the direction of the field and the spiral pattern.

Why, then, does the field become disordered? The answer is that the inner regions of M83 contain unusually large numbers of massive stars, the formation of which was triggered at least in part by the compression of gas in the bar-driven shocks. Massive stars pump energy into the interstellar medium through their powerful winds and eventual explosions as supernovae. The deposition of this energy

and momentum blows out cavities, or bubbles, in the interstellar medium. Spatial clustering of these stars enhances this effect.

The resulting superbubbles significantly distort the ambient magnetic field by sweeping it up into a thin shell⁷. A superposition of these cavities in regions of massive star formation naturally accounts for the fluctuations in the field inferred from the polarization data. The rate of star formation is lower in the spiral arms, so there field amplification by the shock is the dominant effect.

The high rate of star formation in the central regions of M83 has interesting implications for dynamo theories of the origin of the magnetic field. It has been pointed out⁸ that the rate of magnetic field generation increases as a high power of turbulent gas velocity, and the effect of interstellar bubbles on the generation of fields has recently been calculated explicitly⁹. It is also known that dynamos in systems with strong differential rotation produce magnetic fields that are predominantly parallel to the rotational flow, whereas dynamos in which turbulence is strong produced fields that are much more chaotic. It is tempting to speculate that if there is a dynamo operating in the inner parts of M83, then it is (i) vigorous, because of kinetic energy injected by massive stars, and (ii) dominated by turbulent motions rather than by the weak differential rotation which characterizes the inner parts of spiral galaxies.

Even if this picture can be fleshed out and made plausible there are other interesting issues. The mean magnetic field strength deduced is rather large, corres-

ponding to a combined magnetic and cosmic-ray pressure more than four times that measured for our Galaxy. It is generally accepted that the total pressure in these components cannot exceed the pressure of the interstellar gas (which is effectively turbulent pressure; the thermal pressure is about an order of magnitude smaller). The reason is that magnetic fields, cosmic rays, and turbulent pressure all support the gas in the direction perpendicular to the galactic plane. If the gas is supported much beyond its natural scale height, it becomes unstable to Rayleigh–Taylor-like overturning¹⁰ over a few freefall times, or a few tens of millions of years.

The starburst phase itself is presumably shortlived, so we may be observing M83 in a transient and unstable state. This instability problem may be most acute in the outer regions of M83 where magnetic fields are observed but the surface density of the gas is evidently very low. It would be interesting to know whether the gas pressure in M83 is also several times larger than local values for our Galaxy.

Finally, there is the possible detection of a well-ordered field above the disk. Magnetic field could be carried above the disk by expanding superbubbles; the X-ray observations in fact suggest a halo of hot gas. But such a field would probably be rather chaotic. A dynamo¹¹ could produce a well-ordered field, as could an organized flow such as a galactic wind.

Maps of galactic magnetic fields such as those of M83 appear at present to be better able to fuel speculation than to control it, and we are a long way from saturation. Detailed radio maps combined with analyses of the dynamical and other properties of the interstellar gas should refine our picture of the role of magnetic fields in galaxies. On the theoretical side, more realistic models of gas flow in galaxies and of dynamos are needed for meaningful comparison with observations. □

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Muscle transfection made easy

Terence A. Partridge

AT first sight, skeletal muscle is not an obvious tissue on which to attempt gene therapy — it is highly organized and stable, and the nuclei of mature muscle fibres do not undergo division, thus giving no opportunity for genomic integration of exogenous DNA. Last year, however, to general surprise, it was found that simple injection of circular plasmid DNA containing a reporter gene led to a long-term expression of that gene within a low proportion of fibres¹.

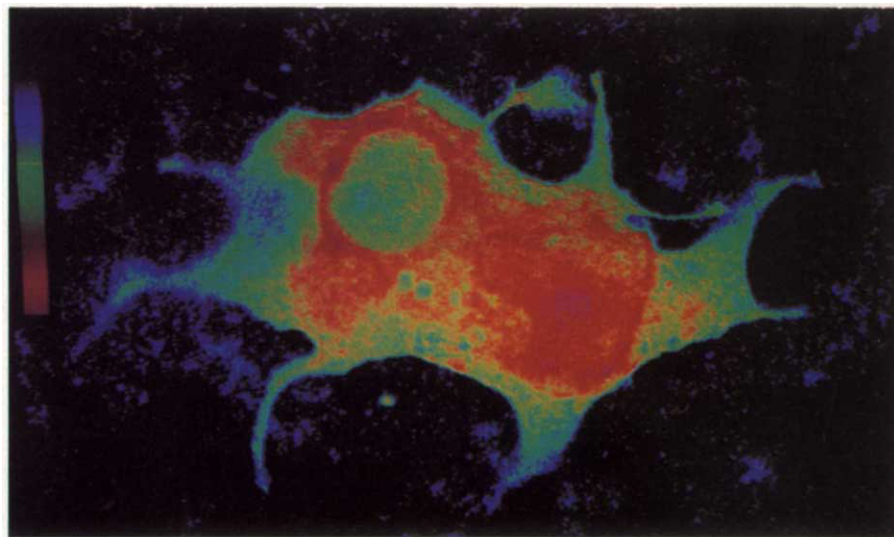
This same procedure has now been applied to insertion of the very large gene which is defective in Duchenne muscular dystrophy² into skeletal muscle of the mdx mouse, a genetic equivalent of Duchenne muscular dystrophy. On page 815 of this issue³, Acsadi *et al.* report that injection of plasmids containing the 12-kilobase or 6.3-kilobase complementary DNAs coding for competent versions of the gene leads to expression of its protein product, dystrophin, in about 1 per cent of muscle fibre profiles. Yet more surprising is the longevity of this effect. Although the study reports on dystrophin expression for only a week after injection of the plasmid, the earlier work demonstrated stable expression of reporter genes for several months, apparently from plasmids which remain circular and episomal (independent of the chromosome) in the non-replicative environment of the muscle fibre. John Wolff, the initiator of this work, says that expression still remains stable after one year (J. Wolff, personal communication).

As it stands, the technique for inducing synthesis of dystrophin within muscle fibres is of great interest as a potential therapy for recessively inherited myopathies such as Duchenne muscular dystrophy. But such notions must be treated with reserve, pending a dramatic improvement in the proportion of muscle fibres which can be induced to synthesize dystrophin. The overall inefficiency is breathtaking — injection of 400 µg of dystrophin cDNA, equivalent to the total genomic dystrophin gene copies of several mice, leads to the synthesis of detectable dystrophin in only some 50 fibre profiles out of 5,000 in a single muscle. A therapeutic effect would require an increase of 10–50 fold in dystrophin-positive fibre profiles. This might be attained by increase in the efficiency of transfection or by 'gene-targeting'. Given such an improvement, the production of large amounts of plasmid DNA is a relatively trivial matter. The widely used replication-defective retroviral vectors are unable to accom-

modate the full length dystrophin gene (although they could carry the 6.3-kilobase 'minigene'⁴) but other viruses, such as adenoviruses, which are being tested for carrying the gene for α 1-antitrypsin into cells lining the lung⁵, may be of value. The possibility of encouraging uptake of genes into muscle fibres by way of specific cell-surface

large areas⁷. These myoblasts also have the advantage of providing new sources of cells for formation of new normal muscle fibres and repair of dystrophic muscle fibre⁸ within muscles where the endogenous repair processes are failing⁹.

The question arises as to whether the dystrophin produced in muscle fibres as a result of direct gene transfection is functional. Much but not all of it is located in its normal position at the cell surface^{10,11}. A high proportion of such surface-stained fibres appear to be normal, with the nucleus in a peripheral



Vivid expression — a colour-enhanced confocal image of a transfected mouse 3T3 cell, showing the expression, in red, of human dystrophin.

receptors is also worth examination. By whatever means the constructs are introduced into muscle fibres, it is to be hoped that the stability of expression of the directly transfected constructs would continue to apply.

It is encouraging that the same technique can be used to transfect the dystrophin gene into cardiomyocytes of the mdx heart, thus providing a potential treatment for the cardiomyopathy of Duchenne muscular dystrophy. In this respect, direct transfection is superior to the other main method being tested for introducing the dystrophin gene into dystrophic muscle, namely myoblast transplantation^{6,7}. No means are currently known for inducing normal heart myocytes to proliferate. And even if we could inject large numbers of such cells into hearts, they would not be able to share their dystrophin with the existing heart cells because, unlike skeletal muscle precursors, heart myocytes do not fuse to form a syncytium.

In skeletal muscle, for the time being, injection of myoblasts obtained from normal mice into the muscles of mdx mice is far more effective than direct transfection at producing dystrophin-positive muscle fibres — injection of 100,000 cells gives up to 30–40 per cent dystrophin-positive fibres, spread over

position, whereas most of the dystrophin-negative fibres carry their nuclei in a central position, indicating that they have probably degenerated and regenerated⁸. But until the yield of positive muscle fibres is increased, it will be difficult to determine whether this association is due to the protection of muscle fibres by dystrophin or to loss of dystrophin expression by fibres which had degenerated and regenerated during the seven days since transfection.

Irrespective of its therapeutic value, direct gene transfection into muscle fibres will surely become a preferred method for preliminary assessment of intramuscular expression of DNA constructs containing muscle-gene promoters; it is cheaper and faster than analysis in transgenic animals, and more relevant to normal physiology than expression in tissue culture. Transplantation of myo-

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G. Acsadi

blasts transfected *in vitro* offers an intermediate option in terms of cost, time and yield of transfected tissue.

One of the more fascinating aspects of the phenomenon is the implication that the skeletal muscle fibre lacks a mechanism for eliminating these episomal DNA constructs. It seems strange that such a GLOBAL EXTINCTION

cosy ecological niche is not regularly exploited by DNA viruses as a lifelong home — or is it? □

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Acid rain at the K/T boundary

Martin Palmer

THE events that ended the Cretaceous period and started the Tertiary, 66 million years ago, have been a source for much study and not a little wild speculation. Of particular interest has been how a large proportion of all species, most strikingly the dinosaurs, met their demise then. Russell¹ posed the question in a slightly whimsical manner when he envisaged the last dinosaur either crawling at its last gasp towards an ever-retreating sea or disappearing in a hurricane of iridium-laden dust and roasted dinosaur flesh. On a more serious note, models have been presented suggesting sudden global warming, something like a nuclear winter, and/or showers of toxic acid rain as agents of destruction. Either increased global volcanism or an extraterrestrial impact would be responsible for these extreme atmospheric conditions²⁻⁴. The emphasis here should be on the word models, as there has been a marked dearth of evidence in the geological record for any of these hypotheses. But Martin and Macdougall now suggest⁵ that a large and sudden change in the strontium isotope ratio of seawater at the Cretaceous/Tertiary (K/T) boundary is evidence of rapid addition of radiogenic strontium to the oceans from the continents resulting from enhanced weathering by global, highly acidic rain.

The ⁸⁷Sr/⁸⁶Sr ratio of the oceans is largely controlled by the relative fluxes and isotope ratios from submarine hydrothermal activity (⁸⁷Sr/⁸⁶Sr = 0.703) and river inputs (⁸⁷Sr/⁸⁶Sr = 0.712). The ratio is recorded by microfossils that make up carbonate sediments, which reveal a gradual increase from 0.70755 80 million years (Myr) ago to 0.70785 just before the K/T boundary. At the boundary there is a sharp increase to 0.70793 followed by a slower fall to 0.7077 about 55 Myr ago. Fluctuations in the marine strontium isotope record as a result of variations in the magnitude and isotope ratios of hydrothermal and riverine fluxes are well documented in the Cenozoic (the past 66 Myr), but the rate of increase at the K/T boundary is exceptionally rapid and the shape of the spike suggests a rapid injection of radiogenic

strontium to the oceans followed by a slower return to the background levels. What then are the possible causes of this spike?

Martin and Macdougall conclude that the most likely cause was a dramatic increase in weathering rates following generation of nitric acid rain by atmospheric reactions between water vapour and nitrous oxides. These reagents would have been produced by shock heating of the atmosphere by the impact of an extraterrestrial bolide. The highly acidic rain, as well as delivering radiogenic strontium to the oceans, released additional CO₂ during weathering, raising temperatures and prolonging the effects of the acid rain.

Direct addition of strontium to the oceans from the bolide or associated impact ejecta would not have provided sufficient radiogenic strontium to yield the observed spike. Martin and Macdougall also reject increased volcanic activity as the source of the additional strontium on the grounds that volcanism at the K/T boundary was dominated by formation of the Deccan basalts in southern India which have ⁸⁷Sr/⁸⁶Sr ratios similar to the hydrothermal input.

In contrast, Javoy and Courtillot⁶ suggest that development of the Deccan traps resulted in extensive melting of the continental crust. This produced explosive acid volcanism that preceded eruption of the basalts and would have delivered radiogenic strontium to the oceans by their weathering and river transport or directly by dumping large volumes of ash in nearby seas. In addition, acid-forming gases released during intensive volcanic activity would also have accelerated the rate of continental weathering. Unfortunately, evidence of these proposed extensive acidic volcanics is thought either to be buried below the Deccan traps or to be weathered away. Of course this in itself does not disprove their hypothesis, but the lack of hard evidence is not particularly satisfying.

Part of the reason Javoy and Courtillot developed their hypothesis was concern in placing the strontium isotope spike right at the K/T boundary. Uncertainties in the age assignments of micro-

fossils analysed for strontium isotopes arise because carbonate sediments are not conducive to direct radiometric dating. Instead age assignments are based on magnetostratigraphy, correlation of palaeontological zones and interpolated sedimentation rates. These techniques have an absolute accuracy of only 0.5–2 Myr for individual samples leading some to propose that the isotope anomaly actually occurred before the K/T boundary and was due to an increased riverine flux related to late-Cretaceous sea-level regression⁷.

Nelson *et al.*⁸ have also suggested that a rapid change in marine strontium isotope ratios occurred before the K/T boundary, although doubts have been raised about their stratigraphy and age assignments (J. A. McArthur, personal communication). The far more detailed study by Martin and Macdougall also reveals an increase in seawater ⁸⁷Sr/⁸⁶Sr ratios preceding the larger spike at the K/T boundary itself, but they ascribe this to normal fluctuations in the relative inputs from river and hydrothermal sources rather than to any special event. Even in this careful study, sceptics could argue, the combination of analytical errors in determining the strontium isotope ratio and imprecisions in the age assignments could mean that the sharp increase in ⁸⁷Sr/⁸⁶Sr ratios at the K/T boundary may actually be less intense and spread out over a longer period than suggested by the data, and simply represents part of the normal fluctuations established before the boundary.

Our present inability to obtain more accurate absolute ages for carbonate microfossils means that we will not be able to resolve fluctuations in the marine isotope record to a much finer degree than already achieved in these recent studies. Until this problem is solved it is unlikely that this record will provide conclusive evidence about events around the K/T boundary, but the attempt to address the problem in a novel manner and by collecting real data is a welcome alternative to the large number of arm-waving, computer-generated models that have characterized much of the debate so far. □

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Curious cortical change

Mike Calford

A REPORT in *Science* by Pons *et al.*¹, describing a massive reorganization in the somatosensory cortex of monkeys with long-term loss of all sensory nerves of the forelimbs, brings into question one of the basic concepts of neuroscience. That concept is the largely stable nature of the adult brain. Pons and colleagues demonstrate an increase in the extent of plasticity from a generally accepted and explicable 1–2 mm to an inexplicable 10–14 mm.

Using electrophysiological recording, the authors mapped the expansion of the representation of nerve impulses from the lower face into cortex that would, but for severance of the forelimb sensory nerves at the dorsal roots (segments C2 to T4), have represented the arm and hand. Although changes of this magnitude have previously been described in developing animals, they have been attributed to, and depend upon, disruption

of the normal processes of neural growth and development. The cessation of these processes defines the adult state.

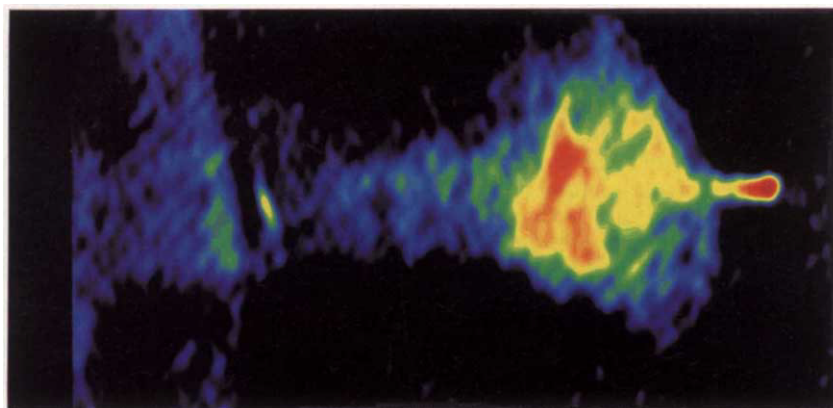
Before the appearance of the paper by Pons *et al.*, the study of plasticity of cortical representations in adult mammals operated largely under a consensus. Different groups working on various species and representations agreed that the limit on plasticity seemed to correspond to the extent of thalamocortical axon arborizations — 1 to 2 mm across the cortical surface (for review see ref. 2). Thus, peripheral denervations that were represented by areas exceeding this limit, such as 5° laser lesions of the retina or amputation of two fingers, left some unresponsive cortex. Smaller denervations in somatosensory, visual or auditory modalities produced, in cortex studied some weeks later, an expansion of adjacent representations into the

denervated area. The basis for these expansions appears to be the normal spread of input from the thalamus³, and such inputs can be readily demonstrated by disruption of intracortical inhibition with pharmacologically active agents⁴. Disinhibition resulting from a small denervation is almost immediate⁵, has a transitory bilateral component⁶ and is followed by a period of one or two weeks in which large receptive fields shrink through a process probably involving some form of synaptic plasticity⁷. The resulting reorganized area of cortex, although remarkable, was not evidence enough to challenge the dogma that the adult central nervous system is basically static, for no neuronal growth was required to explain the phenomenon.

The new finding which upsets this consensus story comes from a partially unplanned experiment. The animals used were originally the research subjects of Edward Taub at the Institute for Behavioral Research in the United States. In 1981, they were seized by animal rights activists, resulting in a change of experimental plan and a 12-year period between deafferentation (severance of the nerves) of one or both arms and the electrophysiological mapping of somatosensory cortex. The role that the long period of denervation played in the generation of the massive plasticity cannot be assessed because no comparable study has been undertaken following shorter periods of deafferentation. It is also unlikely that any such study will be undertaken. The specialized animal care would be prohibitively expensive for most laboratories and the risk of a confrontation with activists would be high. Further experiments to resolve the time-dependence of the process may have to rely on the study of injured animals (assuming that long-term amputees are also found to show massive reorganization in cortex). This is an important issue, because the formulation of a model to study significant long-term plasticity is one of the main aims of this area of research.

No mechanism has been put forward to account for the phenomenon. Pons *et al.* suggest that subcortical relays may be involved; but although plasticity has been demonstrated at lower levels of the pathway⁸, the scale of the changes cannot be accounted for, particularly since the expanded representation of the face is innervated through the trigeminal nerve whereas the deafferented arm is innervated through the spinal cord. Work on the digit representation in the cortex of raccoons suggests, however, that connections between the many somatosensory fields in the cortex may provide an explanation. In concert with the development of dexterity, the raccoon has evolved a greatly enlarged

Supernova debris gets ahead of itself



SOLVING a minor mystery of radio astronomy, on page 785 of this issue, D. A. Frail and S. R. Kulkarni suggest that the curious fan-shaped radio source G5.4-1.2 is the product of a supernova remnant that has overtaken itself. As can be seen from the radio map above, the object bears a striking resemblance to the result of a bullet passing through a sheet of material. The bullet in this case is the pulsar PSR1757-24, which is in the knot of emission at the right-hand end of the nebula. The sheet is a wall of gas and dust swept up from the interstellar medium by ejecta from a supernova explosion on an old, massive star. The pulsar, Frail and Kulkarni argue, was created in the same explosion and received such an enormous kick that it has now overtaken the expanding gas shell. From the current geometry of the shell and pulsar, it is possible to work out where both originated (far beyond the left of the image). Pulsars also bear a useful clock from which their age can be deduced — the rate at which their pulsations slow down. From this, Frail and Kulkarni show the pulsar to be only 16,000 years old, in which case it must have been born in a highly asymmetric supernova explosion that accelerated it up to a speed of over 2,000 km s⁻¹, far faster than any other known pulsar. If so, it should be possible to see the pulsar's advance over the next few years. This would also explain why so few supernova remnants are now associated with a pulsar; the Crab nebula and pulsar, only 900 years old, make a notable exception. In the meantime, the entanglement of the young and vigorous pulsar's magnetic field and radiation with the nebula, expanding at only a slightly slower rate, gives radio astronomers another fine view. □

Cool radiators

P. R. BERGER *et al.* (*Appl. Phys. Lett.* **59**, 117–119; 1991) have succeeded in combining in a single chip the elements of a laser and an electrical thermostat operating on the Peltier effect (the reverse of the thermoelectric effect by which currents are produced from a temperature gradient). In part this is to stabilize the laser's emission frequency, which depends on its temperature. But Berger *et al.* show that varying the temperature with the internal cooler also tunes the frequency over a range of 12 Å. They envisage that such tunable laser chips could be used in high-rate data networks, optical logic switches and spectroscopy.

Glue worms

ENVIRONMENT-conscious do-it-yourself enthusiasts searching for the ultimate superglue need look no further. For there is a glue that withstands pressures of 260 atmospheres, temperatures of 250 °C and sulphurous stews of toxic chemicals — and it is perfectly natural. It is the collagen that keeps deep-sea hydrothermal vent worms from falling apart. But as F. Gaill *et al.* show (*J. molec. Biol.* **221**, 209–223; 1991) there is much to learn about the properties of worm glue. Large amounts of the amino acid hydroxyproline are often thought to confer heat resistance on collagen, but little is present in the remarkably heat-stable collagens from the giant vestimentiferan worm *Riftia pachyptila* and the annelid *Alvinella pompejana*. Perhaps, the authors suggest, there is too little oxygen in the abyss to sustain the hydroxylation of proline residues, or perhaps the high pressure is responsible.

No nothing

WHILE many nuclear laboratories are pursuing the newly identified 17-keV neutrino, K. You *et al.* have been patiently trying to make no neutrinos at all (*Phys. Lett.* **B265**, 53–56; 1991). Like those embarked on searches for the exotic heavy variety, these researchers are hoping to reveal new subtleties of the nuclear weak force. The typical source of neutrinos is nuclear β -decay in which one neutrino and one electron is born. But, depending on whether neutrinos are their own antiparticles or not (whether they are 'Majorana' or 'Dirac' particles), it may be possible for nuclei to decay by the emission of two electrons accompanied by no neutrinos. Searches for such decays have so far been unsuccessful. You *et al.* realized that the nuclide ^{48}Ca should give a distinct signature and so increase their chance of success. Nevertheless, after watching 43 g of the isotope in 37 kg of natural CaF_2 crystals for 7,588 hours in the bottom of a coalmine, they are still waiting for the first event.

cortical representation of the digits — a single digit can occupy an area as large as the entire arm and hand representation in a monkey. If one of the four digital nerves is cut, plasticity is consistent with the underlying spread of thalamocortical afferents described in other species⁹. If all digital nerves are cut, however, a distinctly different type of response is unmasked. In the short term, this takes the form of unusual off- and inhibitory-responses to stimulation of other digits, as well as the presence of many unresponsive neurons. But in longer term experiments the percentage of typical responses increases¹⁰. The source of these responses seems to be existing connections from other somatosensory areas of cortex which do not have the same degree of precision in the topographic representation of the body surface topography as the primary field.

In monkeys, corticocortical inputs to primary somatosensory cortex are extensive and are not as topographically precise as are inputs from the thalamus. If

MOLECULAR FILMS

Laying down organic metals

Martin R. Bryce

OVER the past two decades, molecular charge-transfer salts which conduct electricity (organic metals) have been intensively studied in the form of single crystals¹. But, as discussed at a recent meeting*, these materials have now been fabricated into thin films by vapour deposition techniques without losing any of their remarkable properties. In this form, they will be much easier to exploit in electronic applications.

A charge-transfer (CT) salt is formed by combination of an electron-donor molecule with an acceptor molecule. Traditionally these salts are electrical insulators but many systems are now known in which stacking of the ionized molecules in the crystal lattice occurs in such a way that extensive electron delocalization can occur. The result is high electronic conductivity at room temperature, $\sigma_{\text{RT}}=10\text{--}1,000\text{ S cm}^{-1}$ (for comparison the conductivity of copper metal is 10^6 S cm^{-1}), and even bulk superconductivity at very low temperatures (below 12 K) in a few cases. The donor molecules are typically planar (or nearly planar) sulphur heterocycles which have low ionization potentials, including TTF or BEDT-TTF (see figure), and the counterion is an inorganic anion, such as I_3^- , PF_6^- , ReO_4^- or a more exotic species, such as $\text{Cu}[\text{N}(\text{CN})_2]\text{Br}$ (ref. 2). A striking difference between these organic metals and inorganic metals is that the

corticocortical connections are responsible for the unexpected plasticity shown by the Taub monkeys, then it will still be possible to hold to the idea that adult brains are the stable product of their development — and avoid the inference that the generation of new neuronal projections is involved. □

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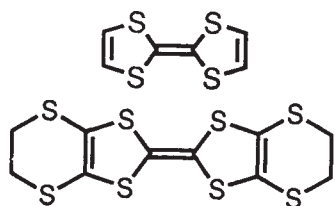
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former are highly anisotropic; that is, high conductivity occurs along only one (or occasionally two) of the crystallographic axes.

For the many technical applications envisaged for organic metals, far more attention has been given to electrically conducting polymers (polyacetylene and polyaniline, for example), which are relatively easy to prepare in the form of free-standing thin films^{3,4}, than to CT salts. Rechargeable solid-state batteries, electrolytic capacitors and electrochromic devices, such as 'smart windows' have been developed using these polymers. The frailty of the crystals of molecular CT salts has imposed a severe limitation on their practical usage. It is recognized, however, that if this problem can be overcome, CT salts may offer distinct advantages over polymers. First, there is greater scope for fine-tuning their properties by chemical modification of the starting materials. Second, CT salts are usually stable in air, in contrast to many conducting polymers which readily decompose. And lastly, the most glamorous property, organic superconductivity, has been found only in CT salts, not in polymers (presumably this is at least partly because of inherent disorder found in the latter systems). It is, therefore, an exciting step forward that stable thin films of CT salts can now be fabricated using vapour deposition techniques.

Several semiconducting thin films of

*Molecular Conductors for Information Technology, Sesimbra, Portugal, 23–25 May 1991.



Structures of (top) tetrathiofulvalene (TTF) and (below) bis(ethylenedithiolato)-TTF (BEDT-TTF), sulphur-based heterocycles that could be found in microchips of the future.

CT salts have been fabricated over the past few years using the Langmuir-Blodgett technique⁵, the deposition of molecular films from solution. However, although these films have unveiled all kinds of interesting chemical and physical effects, they are not so suitable for use in electronic devices; their thermal and mechanical stability tends to be rather low and the technique requires the synthesis of amphiphilic molecules (combining hydrophilic and hydrophobic parts) which is not always so easy. It is now clear that vapour deposition can provide more robust films with higher values of conductivity than Langmuir-Blodgett films. Two Japanese groups have developed this technique to obtain metallic films of TTF⁶, and superconducting films of BEDT-TTF⁷, incorporating iodide anions.

In new work⁶ described by M. Yudasaka (Hoechst, Japan), tetrathiofulvalene (TTF) and iodine are co-evaporated from separate crucibles in a pyrex-glass chamber under high vacuum (3×10^{-5} torr). Deposition of the resulting TTF iodide CT salt is achieved on a glass substrate. The temperature of the crucibles and the deposition substrate is important in determining the chemical composition of the deposited film. Under the optimum conditions (TTF source, 50–70 °C; iodine source, 0 °C; and substrate glass, 0–20 °C) TTF-I_{0.7} was deposited as a thin film between 0.1–1 µm thick. With the substrate temperature below –20 °C, the films were contaminated with uncomplexed TTF, and above 30 °C nothing was deposited.

Ultraviolet spectra of the metallic film show that the TTF molecules have been oxidized by the iodine to form TTF radical cations which can provide the charge carriers. Polarized spectra show marked dichroism, providing evidence that the molecules are well oriented within the film. Consistent with this structure, anisotropic electrical properties were observed with a room-temperature conductivity value along the most highly conducting axis of 10^2 – 10^3 S cm⁻¹. A transition to a less conducting state occurs at about 200 K. Overall, these data are very similar to those of single-crystal samples of TTF-I_{0.7}.

K. Kawabata and coworkers⁷ (Idemitsu Kosan Co.) have fabricated superconducting thin films of the CT salt formed by BEDT-TTF and iodine using basically the same technique, although experimental details are quite different. In these experiments, the raw material was heated to 200 °C to achieve sublimation and deposition onto a potassium chloride substrate kept at 70 °C. The raw material was either BEDT-TTF powder which was doped by reaction with iodine during deposition (as described above for TTF iodide) or, alternatively, preformed BEDT-TTF iodide salt (prepared electrochemically) was used. Both methods gave films with essentially the same quality with a typical thickness of 500 nm. The separation of the crucible and the substrate is important, 3 cm being the optimum distance.

X-ray diffraction data for the evaporated film fit well with the α -phase of (BEDT-TTF)₂I₃ crystals which would indicate a high degree of orientation in the film. The film's in-plane conductivity is 1 S cm⁻¹ at room temperature and decreases with decreasing temperature, showing the material to be semiconducting. Single crystals of the α -phase of (BEDT-TTF)₂I₃ can be transformed thermally into the α_1 -phase which is a stable superconductor⁸ with $T_c = 9$ K. This α_1 -phase was obtained from the films when they were annealed at 90 °C for 40 hours in air, along with a very small amount of BEDT-TTF resulting from decomposition of the salt. Magnetization measurements established that the annealed films undergo a superconducting transition at about 5 K. The critical temperature is very sensitive to the evaporation conditions, with the variation seen in T_c probably arising from disorder in the crystalline regions of the film.

It is to be expected that a wide range of conducting and superconducting CT salts will form well-ordered thin films using the above evaporation techniques, and hopefully materials with higher T_c values will be obtained. Indeed the recent discovery⁹ of superconductivity at 18 K in evaporated films of potassium-doped fullerene C₆₀ should add impetus to the study of thin films of CT salts. □

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Life after death

THANKS to the polymerase chain-reaction, antique DNA can now be detected in all sorts of long-dead fossils and museum specimens. Some of it is 20 million years old. How can this be, when DNA ought to hydrolyse completely in a mere 10 thousand years?

Daedalus sees it as just another aspect of the ruthless survival strategy of DNA. In 'selfish gene' terms, it is parasitic upon the creatures it inhabits. It gives them abilities and instincts which drive them to replicate it as copiously as possible in the next generation before they die.

Now the death of its host is certainly a setback to any parasite. To some (like the tapeworm) it is fatal; but smarter ones (like the louse) simply go off in search of a new host. DNA, the smartest parasite of them all, is most unlikely to be defeated by the death of its host. In its relentless struggle to survive and reproduce, it must have evolved the capacity to withstand the chaotic chemistry of death. As the body around it decomposes or fossilizes, it somehow contrives to keep itself largely intact — even over 20 million years.

This, of course, is only half the battle. Sooner or later the DNA has to get back into the mainstream of life. It has to infect a living host, and start replicating again. Now, DNA is rather good at forming microscopic infective particles such as viruses and plasmids. So Daedalus argues that it must escape from dead tissue in the form of fertile plasmid-like spores each containing a few genes. These thereafter drift around the environment. Every so often, such a spore will chance to be present at an act of sexual fertilization. It may then be taken up by the ovum along with the sperm, and can start a new genetic career.

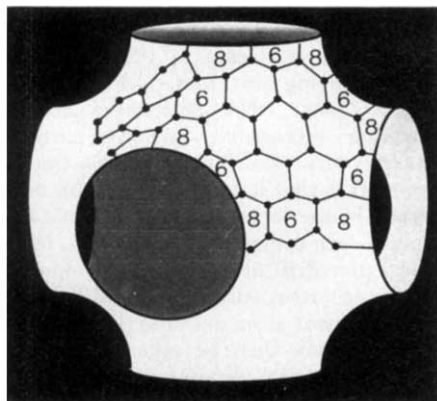
There is no guarantee, of course, that it will find itself in a host of the same species, or that its insertion into the genes of that host will make any sense or do any good. Such random insertions, mainly deleterious or useless but occasionally beneficial, are of course well known. They are called mutations.

On this theory, DNA has a life-cycle as chancy and fantastic as that of any other parasite. Its spores must be everywhere. The DNA already on the replication bandwagon must mount an immune response against them, so successful mutations are quite rare. To test his challenging theory, Daedalus is mating mice, fruit-flies, and so on, in unhygienic surroundings rich with plant and animal remains, to compare their mutation rates with those of creatures mated in a scrupulously DNA-free clean room. Positive results will open up a whole new technique of genetic engineering. DAVID JONES

Diamond from graphite

SIR — Daedalus is preternaturally sensitive to the scientific atmosphere. Having predicted¹ the larger buckminsterfullerenes — cages of graphite-like sheets, with 12 five-membered rings among the hexagons to permit topological closure into a shell — he now goes on to consider graphite foam². We have recently been building models of periodic minimal surfaces³ from graphite-like sheets. Because minimal surfaces have zero mean curvature and the gaussian curvature (the product of the two principal curvatures; one positive and one negative for a saddle-shaped surface) is everywhere non-positive, it is necessary to introduce rings of more than six carbon atoms (for sp^2 bonding). We substitute a few rings of eight for hexagons and find that this can be done with surprisingly little strain. The octagons are puckered with bond angles close to 120° , and sit appropriately on positions of symmetry $4m$.

The simplest surface is shown in the figure. The P-surface has been covered with a graphite net of 12 octagons and 80 hexagons. There are 96 asymmetric units



The periodic minimal surface, called the P-surface, of H. A. Schwarz decorated with a graphite mesh. This unit cell, of space group $Im\bar{3}m$, repeats by translation. Rings of eight allow the gaussian curvature to be negative, that is, saddle-shaped.

in the cell shown, which has a repeat distance about ten times the bond length. The mean coordination number is 6.26.

The diamond D-surface of H. A. Schwarz has been built with 8 tetrahedral units per cell, so that there are 768 carbon atoms per face centred cubic unit cell. It uses the same $30^\circ - 45^\circ - 90^\circ$ patches as the P-surface illustrated. Here they are tetrahedral joints, where four tubes meet tetrahedrally, each consisting of six octagons and 40 hexagons so that the mean coordination number is also 6.26. (The mean coordination number for a plane net must be 6 and for the spherical shell of buckminsterfullerene,

which has a positive gaussian curvature, it is 5.625.) The same tetrahedral units may also be joined hexagonally. Thus, paradoxically, we can make the diamond structure out of graphite.

The P-surface of Schwarz has also been built with 432 atoms per cell in 200 hexagons and 12 octagons using a larger patch, so that the mean coordination number is 6.11. Here the unit cell edge is about 15 times the bond distance.

In each case a plane triangle from the graphite net with angles $30^\circ - 60^\circ - 90^\circ$ is curved so that 60° vertices become 45°

and eight fit together to give an octagon. The triangle can be chosen in several ways, and other surfaces can be similarly decorated.

Thus, we find that a variety of ordered graphite foams look quite possible. The question is how to synthesize them.

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Mitochondrial protein charge

SIR — Most mitochondrial proteins are imported from the cytosol and are targeted into the organelle by an amino-terminal presequence which is removed during the importation process^{1,2}. Within the organelle they are subject to specific local conditions, such as higher pH and higher calcium-ion concentration than in the cytosol, and to interactions with a specific subset of cellular proteins. Thus, the question arises of whether evolutionary pressure has endowed mitochondrial proteins with specialized structural features. The observation that the authentic precursor of mitochondrial aspartate aminotransferase is imported four times faster than a chimaeric construct consisting of the same presequence fused to the much less basic homologous cytosolic isoenzyme³ sug-

gests that the pI value could be such a compartment-specific trait. The pIs of both mitochondrial and cytosolic proteins vary considerably (see refs 4,5), making comparisons difficult. But by comparing pIs in pairs of similar mitochondrial and cytosolic isoproteins, we find that, with few exceptions, the pI of the mitochondrial isoprotein is higher than that of its cytosolic counterpart, the average difference being 1.4.

The average pI values of the mitochondrial and cytosolic isoproteins are 7.2 and 5.8, respectively (see table). Seven of the eleven isoprotein pairs show the corresponding positive pI difference (ΔpI) unambiguously (regardless of the analysed species or tissue). Identical pIs were reported for phosphoenolpyruvate carboxykinase from chicken

ISOELECTRIC POINTS OF HOMOLOGOUS MITOCHONDRIAL AND CYTOSOLIC ISOENZYMES

Enzyme	Source	pI of isoenzyme:		Difference
		mitochondrial	cytosolic	
Aconitate hydratase	Pig heart, liver, kidney	7.5	5.4	2.1
Adenylate kinase	Pig heart	4.7–7.5, 9.3	4.7–7.5, 9.3	0.0
	Rat liver	8.0	7.5	0.5
Alanine aminotransferase	Bovine brain	7.2	5.2	2.0
	Pig liver, kidney	8.0–9.0	5.3–6.0	2.9
Aldehyde dehydrogenase	Sheep liver	6.6	6.2	0.4
	Sheep liver	5.22–5.76	5.2	0.3
Aspartate aminotransferase	Rat liver	5.6	5.8, 8.5	–1.6
	Human liver	9.6	5.22–5.62	3.2
Creatine kinase	Chicken heart	9.0–9.5	6.7	2.5
	Rabbit liver	9.6	5.3	4.3
Fumarase	Chicken heart, brain	9.3–9.5	7.4, 7.5	2.0
		8.5–8.9	6.1, 6.5	2.4
3-Hydroxy-3-methylglutaryl coenzyme A synthetase	Human fibroblasts	5.65–6.8	5.5, 5.6	0.7
	Chicken liver	7.2	4.8, 6.7	1.5
Malate dehydrogenase	<i>S. cerevisiae</i>	6.8	6.75–7.1	–0.1
	<i>N. crassa</i>	7.0, 7.5	5.6	1.6
Phosphoenolpyruvate carboxykinase	Rabbit heart	9.0, 9.2, 9.5	5.1	4.1
	Pig liver	10	5.1	4.9
Serine hydroxymethyltransferase	Chicken liver	5.0	5.0	0.0
	Rat liver	5.3	4.9	0.4
Average (s.e.)*		7.2 (0.4)	5.8 (0.2)	1.4 (0.4)

The table contains all pairs of homologous mitochondrial and cytosolic isoenzymes with known isoelectric points that we could find. References reporting the pI values and the homology of the two isoenzymes (except for alanine aminotransferase and aconitate hydratase) are available on request.

*Each protein was given equal weight in the calculation by using the mean of the values obtained for different sources. A difference of the means test indicates the mean values of the pIs to be significantly different with $P < 0.02$.

and for adenylate kinase from pig, as opposed to a positive ΔpI for the latter enzyme from rat. A small negative ΔpI of the malate dehydrogenases in yeast contrasts with large positive ΔpI s in *Neurospora crassa* and rabbit. The only significantly negative ΔpI is found in the case of rat liver aldehyde dehydrogenase, but this again contrasts with a positive ΔpI for the sheep liver enzyme.

Depending on the protein, the observed difference in pI is due either to more basic amino-acid residues, or to fewer acidic residues or to both in the mitochondrial isoprotein (data available on request). Comparisons of the amino-acid sequences do not reveal any preferential distribution of the positive and negative charges or of the charge differences between the two isoenzymes along the polypeptide chain.

The evolutionary selection for positive charge in mitochondrial isoproteins probably relates either to facilitated importation into this organelle or to adaptation to conditions inside mitochondria.

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Classical records

SIR — Ian Percival's suggestion (*Nature* **351**, 357; 1991) that DNA provides an example of a classical measurement embodied in a microscopic system raises some questions. It would appear that any expression of the alteration of a DNA molecule requires a considerable amplification. For example, Percival mentions leukaemia, but leukaemia is not a microscopic phenomenon — it is a statistical phenomenon involving a huge number of cells. The result of the decay of a single radioactive atom can be thought of as a microscopic 'record' of a quantum event which can be preserved indefinitely. However, this record remains inaccessible until 'amplified' by an appropriate macroscopic instrument.

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New possibility for planet

SIR — The entirely unexpected nature of the setting in which the new planet in orbit around pulsar PSR1829–10 was discovered¹ has resulted in much controversy about the sequence of events that led to the configuration now observed. It is widely presumed that the supernova explosion that gave birth to the neutron star was a type Ib or type II event, triggered by core collapse in a massive ($M \geq 8 M_{\odot}$) progenitor. This assumption leads immediately to some serious problems, which have been considered by the planet's discoverers¹ and also by Black in *News and Views*².

Confronted by these difficulties, one might resort to any of a number of hypotheses. The planet may have formed after the supernova explosion or, perhaps, it was captured from a passing planetary system. Neither of these is attractive. In the first case, the pulsar is thought to be young, with a characteristic age estimated to be only about 1.25 Myr. This can scarcely be sufficient time to form a planet of mass $M \approx 10 M_{\oplus}$ in an environment which was, in all probability, thoroughly scoured by the supernova blast wave. It is not impossible that the planet was gravitationally captured from a system in transit through the pulsar's neighbourhood, but the required 'fine-tuning' of the encounter parameters makes such an event highly unlikely, especially given the relatively short interval during which it must have occurred. Bahcall³ has raised the intriguing possibility that many, perhaps most, core collapses result in 'neutrino bombs', with very little in the way of an optical display; a nearby planet might survive such a catastrophe, although the short lifetime of the massive progenitor, and its immensity during the red supergiant phase remain causes for concern.

I suggest another possibility, that the supernova explosion which left the remnant PSR1829–10 was of type Ia, and that the progenitor was a single, massive hot white dwarf. An interesting model for such events has been described by Bowers and Deeming⁴, in which hot white dwarfs may exist at masses slightly above the Chandrasekhar limit for zero temperature electron-degenerate configurations because of pressure support from nondegenerate nuclei. For a white dwarf at temperature T , composed of material of mean atomic weight A , they argue that the critical mass M_{Ch} is given by

$$M_{Ch}(T) \approx M_{Ch}(0)(1 + 2 \times 10^{-11} AT)$$

This decreases as the star cools, and, when it falls below the actual mass,

collapse occurs. What ensues is not clear, but Bowers and Deeming suggest mechanisms leading to an explosion which ejects the outer 0.1–0.2 $M_{\odot}$ of the white dwarf, and leaves a neutron-star remnant. The appeal of this in the present context is clear. Because the star whose remnant is the white-dwarf presupernova need not be especially massive ($M \geq 1.5 M_{\odot}$), it has a long lifetime, ample for the formation of a planetary system. Second, such stars do not expand to enormous radii as red giants (50–60 $R_{\odot}$ is typical) and, even if they do so as asymptotic giants, their low-mass envelopes are extremely tenuous. Thus, the orbital decay problem is eased, if not eliminated. Third, the mass of debris incident on a nearby planet after the supernova explosion is sharply reduced, thereby enhancing the planet's prospects for survival. Fourth, the central mass left after the outburst is about 80% of the precursor mass, so that the orbits of companion planets probably remain bound. Moreover, the model accounts naturally for the apparently complete absence of hydrogen features from supernova Ia spectra, which is not readily explained by models requiring mass transfer from the outer layers of a hydrogen-rich stellar companion, or by explosions in a common envelope⁵. At the same time, it obviates concerns about the stability of close planetary orbits in a binary system.

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Ticking off

SIR — It was with alarm that I read in *News and Views* that East Coast fever, which is caused by infection with the protozoan parasite *Theileria parva*, can also be transmitted by mosquitoes (J. Brady *Nature* **351**, 695; 1991). It may be possible to "see tsetse flies from space", as the title of the article concerned has it, but it is clearly not possible to distinguish that ticks (C. P. Lounsbury, *Transvaal Agric. J.* **2**, 4; 1903) rather than mosquitoes are the vectors of East Coast fever.

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Hepatocellular carcinoma mutation

SIR — In hepatocellular carcinoma (HCC), mutations within the p53 gene occur selectively at the third position of codon 249 (refs 1,2), unlike other human cancers where many different codons have been involved (ref. 3 and references therein). The patients in refs 1 and 2 came from areas of the world (South Africa and Qidong province of China) where there is a high exposure to hepatitis B virus (HBV) and potentially a high exposure to aflatoxin B₁. It has been suggested that aflatoxin B₁ is responsible for the observed mutations^{1,2}, but all patients who carried p53 mutations had been or were infected with HBV. HBV infection could have been the cause of these p53 mutations, or more likely, the interaction between HBV and aflatoxin B₁ could be the necessary prerequisite for the generation of these changes in codon 249. It is thus of great interest to determine whether codon 249 mutations occur in people with HCC who have not been exposed either to HBV or to high levels of aflatoxin B₁, or have been exposed to HBV in the absence of aflatoxin B₁.

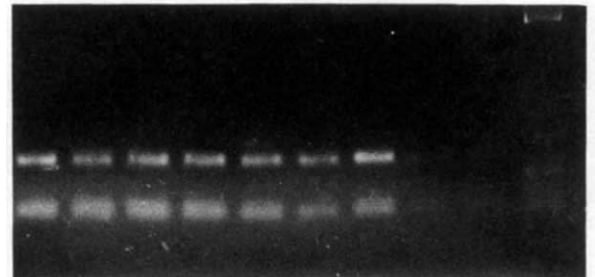
We have screened 16 HCC DNA samples, from patients whose demographic details are listed in the table, for mutations at codon 249 of the p53 gene. Any changes to the second or third bases of codon 249 in exon 7 of the p53 gene result in the abolition of a restriction site for the enzyme *Hae*III (ref. 2). We have combined the polymerase chain reaction

(PCR) with restriction analysis to study possible mutations of this codon in our HCC samples. Oligonucleotide primers (upstream 5'-TCTCCTAGGTTGGCT-CTGACT-3' and downstream 5'-TCCTGACCTGGAGTCTTCCAG-3') were used to amplify a 125-base-pair fragment containing all of exon 7 and part of the flanking introns⁴. We carried out PCR on 100–200 ng of HCC DNA using 50 pmol of each primer, 2 U *Taq* polymerase (Promega) in 1 × *Taq* polymerase buffer (Promega) in a total volume of 50 µl. We performed 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The products were run through 1% Seakem ME (FMC) + 2% NuSieve GTG (FMC) agarose gels and stained with ethidium-bromide.

The 125-base-pair fragments containing exon 7 were excised from the gel and eluted from the agarose by centrifugation⁵. We added MgCl₂ to the eluate to find concentration of 10mM and precipitated DNA by addition of two volumes of ethanol and leaving at –20 °C overnight.

The DNA was pelleted by centrifugation for 15 min, washed once with 70% ethanol, desiccated and resuspended in 7 µl 10 mM Tris, 1 mM EDTA, pH 8. These fragments were then restricted

for 3.5 h at 37 °C with 10 U *Hae*III (Toyobo) in a final volume of 10 µl. The resulting digests were then electrophoresed through 1.5% Seakem ME +2.5% NuSieve GTG agarose gels. Wild-type p53 alleles would result in cleavage by *Hae*III at codon 249, giving products of 83 and 42 base pairs using the primers indicated above. Any changes at codon 249 at position 2 or 3



Nine representative HCC samples digested with *Hae*III. Each shows bands at 42 (lower) and 83 base pairs (upper), indicating total cleavage of the original (unmutated) 125-base-pair PCR fragments. Right-hand lane, marker fragments, generated by *Hind*III cleavage of phage ϕ X174 DNA.

would result in destruction of the *Hae*III site and an uncleaved product of 125 base pairs.

All our HCC samples (see figure) were cleaved by *Hae*III at codon 249 of p53. Thus none of the patients was found to have mutations at this position. Our results therefore support the postulated role^{1,2} of aflatoxin B₁ in the generation of specific mutations of the p53 gene and, in addition, provide evidence to suggest that HBV infection alone does not contribute to these base changes.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

DNA FROM PATIENTS WITH HEPATOCELLULAR CARCINOMA

Patient	Sex/Age	Ethnic origin	Cirrhosis	Histology	Grade	Predisposing factor	Presentation
HBV–							
1	F44	Caucasian	–	pleo	2		early
2	F36	Caucasian	–	trab	1		early
3	M71	Caucasian	–	trab	2		late
4	M67	Caucasian	+	trab	2	haem	late
5	M68	Caucasian	+	pseu	1–3	alco	late
6	M76	Caucasian	+	trab	3		late
7	M79	Caucasian	+	trab	2	haem	late
8	M68	Caucasian	+	trab	2–3	haem	late
HBV+							
9	M51	Melanesian	–	trab	2		early
10	M52	Aborigine	+	trab	1		early
11	M52	Caucasian	+	trab	1		early
12	M29	Melanesian	+	pleo	3		early
13	M57	Vietnamese	+	trab	1		late
14	M35	Melanesian	–	pseu	2		late
15	F69	Caucasian	+	pseu	1		late
16	M36	Caucasian	+	trab	2		late

Histology is as follows: trabecular (trab), pleomorphic (pleo), pseudoglandular (pseu), fibrolamell (fibr).

Tumours were graded as follows: grade 1, well differentiated; grade 2, moderately well differentiated; grade 3, poorly differentiated.

Haem, haemochromatosis; alco, alcoholism.

Time of presentation: early, for example, found incidentally at transplantation or screening for alpha-fetoprotein and tumour less than 2 cm in diameter.

Eight patients had been exposed to HBV and eight had not. None of the patients had accumulated a high exposure to aflatoxin B₁.

Sending smoke signals

Jack Ruina

A Path Where No Man Thought: Nuclear Winter and the End of the Arms Race. By Carl Sagan and Richard Turco. *Random/Century*: 1991. Pp.499. \$27.95, £20.

WHAT have we here? A short book written for a general audience by two of the originators of the 'nuclear winter' theory. In it they describe the horrible environmental consequences of an all-out nuclear war, prescribe ways to mitigate these (not surprisingly by eliminating most nuclear weapons) and then suggest how we might reach that happier situation.

Sagan and Turco are two of the five authors of the *Science* paper published in 1983 that first developed the theory that smoke from many burning cities after a massive nuclear attack might alter climate on a global scale and reduce average world temperatures by as much as 20 °C. The effects on man and beast would be devastating. The theory was based on an analysis by Paul Crutzen and John Birks, published in 1982 in *Ambio*, that suggested that fires generated in a nuclear war could produce sufficient smoke to have substantial effects on the climate. Others have since refined the analysis: the general conclusion now is that the climatic effects are not likely to be as severe as first depicted and that a more apt description of them might be nuclear 'autumn' rather than winter. (Sagan and Turco 'lightly' acknowledge these later analyses.) But experts still concede that if the industrial world should go up in smoke, serious climatic consequences would be likely.

Nuclear-winter analyses are fraught with uncertainty. Just what would an all-out nuclear war be like? Would cities be heavily targeted and, if so, what would be the nature of the consequent fires? How much and what kind of smoke would be generated and how would it be distributed? How much of the solar radiation normally striking the Earth's surface would be absorbed by this smoke and for how long? And then just what would the climatic consequences be? For example, there is an uncertainty of a factor of 50 in the exponent when calculating the average reduction in sunlight, meaning that the effect on sunlight absorption could be anything from inconsequential to one that results in complete blocking, at least for a while.

It is indeed surprising that over a 40-year period of detailed analyses of the many consequences of nuclear war, the long-term climatic effects of the massive fires that might result were not adequately taken into account. So Sagan

and Turco, with their nuclear-winter colleagues, deserve great credit for analysing and drawing attention to the possibility of another horrendous outcome.

But in the language of game theory, neither hawks nor doves ever imagined anything but horrendous consequences — the differences lie in their strategies for avoiding a nuclear war altogether. The hawks believe that to assure deterrence, a vast and complex nuclear arsenal with a wide spectrum of capabilities is needed to convince the enemy that it will lose in every possible nuclear encounter. Doves believe that enough nuclear weapons only to threaten nuclear devastation are needed. It is not a gap in the repertory of imagined and unimaginable consequences that has limited efforts to deal with the threat of nuclear war. Nonetheless, as Sagan and Turco point out, nuclear winter, on the scale that they indicate, would extend the worst of conceivable man-made horrors

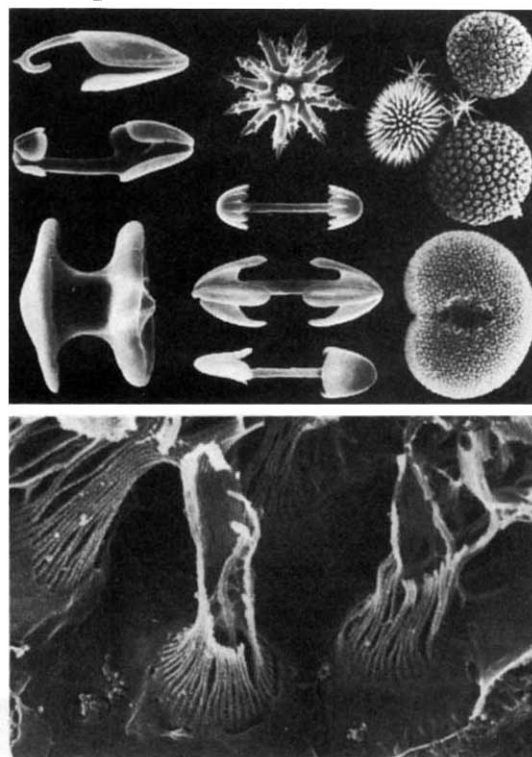
to parts of the world that are not close, either politically or geographically, to the nuclear combatants.

Sagan and Turco begin by describing nuclear winter and the basis of the theory. Then they briefly outline the other consequences of nuclear war: the production of toxic gases from fires, radioactive fallout and ozone depletion. The authors look at all the issues 'through a glass darkly', but the horrors of an all-out nuclear war hardly need a blacker perspective. They do, however, agree that it "seems highly unlikely, that even intentionally, we could destroy all life on Earth", concluding that some insects and grasses would surely survive. (Thank God.) Sagan and Turco then go on to contend that cities and oil-storage sites would be targeted in a nuclear war; if this were not so, nuclear winter would probably not occur.

The rest of the book (more than half) deals with nuclear policy rather than science. Nuclear deterrence is discussed generally: the possible self-deterrent effects of nuclear winter and its effect on noncombatants. Finally, the authors make proposals for reducing the world's nuclear arsenals below the current insane number that we have and below what they believe could cause nuclear winter. Except for being set in the con-

Absorbing creatures

Most people know that sponges come from the bottom of the ocean and make performing one's ablutions more pleasurable. Fewer are aware that they harbour antibiotic and anticancer substances. But how many could decide whether sponges are plants or animals? In fact they are multicellular, typically marine animals that usually occur in complex sessile colonies. Because they are built on a different plan from the Metazoa (for instance, they lack a nervous system), sponges are grouped separately from all other multicellular animals as Porifera, and are presumed to have arisen from unicellular ancestors independently. Their porous body is supported by a fibrous framework reinforced with a mineral skeleton of calcareous or, as shown here, siliceous spicules (top, $\times 1,250$). Water carrying food and oxygen is drawn into chambers lined with choanocytes (bottom, $\times 4,500$). Each of these cells has a flagellum for water propulsion, and a collar of microvilli for ultrafiltration of food particles. The electron micrographs shown here are two of over 100 in the *Atlas of Sponge Morphology* edited by L. De Vos *et al.* With its narrative and line drawings, the volume provides an alluring and masterful guide to the anatomy, physiology and reproduction of sponges, as well as to the remarkable number of microsymbionts that they shelter. Published by Smithsonian Institution Press, price is \$41.95, £27.25. **PT**



text of nuclear winter, the strategic and political thinking here is not particularly new and is now a bit outdated. But this is to be expected when the political world is changing so rapidly and the fundamental concepts of strategic policy are being questioned everywhere.

Sagan and Turco are deeply troubled by the danger presented by our large nuclear stockpiles. Although they claim that they arrived at their policy prescriptions only after being traumatized by the results of their scientific studies of the long-term environmental consequences of nuclear war, the book reads as though it was the other way around — that it was the nuclear arsenals that worried them (quite understandably), the scien-

tific quest being to find out why. The scientific arguments are therefore less convincing than they might otherwise have been.

The basic thesis that Sagan and Turco set out should present them with a dilemma. If indeed the possibility of nuclear winter makes the prospect of an all-out nuclear war even worse to behold, then would more data and analyses demonstrating that the climatic effects would be much less severe, make nuclear war more acceptable? □

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Myths in creation

Richard Mabey

The Naming of the Beasts: Natural History in the Medieval Bestiary. By Wilma George and Brunsdon Yapp. Duckworth: 1991. Pp.231. £42.

THE bestiary is, strictly, a mediaeval phenomenon, a combination of natural description and moral example that had its last echo in the Victorian 'parables of flowers'. But the lives — real and imagined — of animals continue to provide a rich array of raw material on which humans can project their fears and ambitions. The Surrey puma, weeping seal pups, piratical magpies, headlice, rotweillers... our own anthology of totems and scapegoats is still flourishing nearly a thousand years after it was being reported that the basilisk could kill by its looks alone.

The perennial fascination of bestiaries lies in the gap between the fabled creatures and their natural prototypes. What sparked off the process of mythologization? Was it the result of our seemingly deep need to construct hierarchies out of natural creation, or simply due to ignorance and bad observation? Was the widespread bestiary account of lion cubs being stillborn and remaining dead for three days until the male lion breathed life into them a veiled compliment to the king of beasts?

George and Yapp provide half of the answers to these questions. They set out to evaluate how much of the information in bestiaries was based on real natural history. Most of the 40 or so illustrated manuscripts that survive date from between the twelfth and fourteenth cen-

turies (with a handful outside these limits) and were produced in British monasteries for internal use. Their authors and illustrators often had little choice but to rely on earlier, overseas accounts, and so exotic creatures and their fabulous habits were repeatedly copied and rehearsed in much the same way as the formal figuring on icons. Few English monks, for instance, would have set eyes on the sea eagle *Aquila*, yet this bird occurs in all of the bestiaries as an odd amalgam of precise observation and poignant legend. It has unfeathered legs, catches fish (these attributes distinguish



Lupus, the wolf, creeping towards a flock of sheep, tail between its legs.

it from the golden eagle) and can spot its prey from up high. But as it ages, its eyesight grows dim, and it must rejuvenate itself by flying close to the Sun, then having to plunge into a pool to put out its burning wings.

There are many more outlandish stories told in bestiaries: lynxes whose urine turns to stone; partridges partial to buggery; hedgehogs climbing vines to scrump grapes on their spines. But George and Yapp's detailed and sympathetic dissection soon dispels any suspicion that the accounts are simply superstitious ramblings. In the later manuscripts especially, many of the illustrations are beginning to look like first-

hand field drawings. The spoonbill in a Cambridge bestiary of 1450 is, bizarrely, sitting in a tree; but it is a lively and unmistakable sketch of a bird that the monks could well have watched in the local fens. The authors might also have known wolves. There are many remarkably accurate descriptions of the short breeding cycle and hunting techniques of these animals, although there is no mention of their social pack life.

The description of the pelican's life-history (of which few north-European writers could have had first-hand knowledge) suggests how anecdote and rumour could have been turned into myth. One image in almost all the illustrations and texts about *Pelicanus* is that of parent birds dripping blood from their sides into the mouths of their chicks — the symbolic 'pelican in its piety' of later Christian art. Contemporary myths explain this as an act of penitence by the birds for having once killed their chicks in anger. But George and Yapp suggest that the idea may have originated from misunderstood observations of pelicans feeding their young by regurgitation.

George and Yapp are not historians and steer well clear of interpretation. But the more fastidious their speculation, the more it heightens one's curiosity about the unnatural ingredients of the bestiaries. They argue persuasively that

the unicorn may have been inspired by the Arabian oryx, young individuals of which often have only one horn. But is there some kind of sexual symbolism behind the unicorn's almost invariable representation kneeling before a virgin, horn to the fore, while men hack it to pieces with axes? And what about the meaning of some of the bestiaries' other doubtful creatures? George and Yapp suggest that there may be a shadowy king cobra behind the basilisk's baleful stare; that the beguiling cries of monk seals could explain the myth of sirens. But surely some deeper well is being plumbed in the portrayal of the satyr's accoutrements and feisty habits, and in the bizarre mixture of man's face (complete with nightcap), lion's body and scorpion's tail given to the mantichora. And why hybrid animals at all? Structuralists might suspect an engrammed appreciation of symbiosis from their universality. Certainly the cumulative impression given by the documentation in this book is not one of ignorance, but of half-formed ecological instinct. □

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On the beam

O. S. Heavens

The Laser in America 1950–1970. By Joan Lisa Bromberg. MIT Press: 1991. Pp.310. \$30, £26.95.

THE period leading up to the production of the first working laser in 1960 by Theodore Maiman was one of frantic excitement for all groups involved. Tongue-in-cheek forecasts that lasers would be operating at "3.15 p.m. on such and such a date" were liable to be taken seriously.

It is ironic that around this time, graduates with even a modest grounding in optics were in short supply. The Columbia Radiation Laboratory could provide the latest hardware for microwave experiments, but a convex lens was hard to find. Optics had indeed become unfashionable in the physics curricula of universities, a decline that probably began even before the Second World War. Maxwell had 'done' optics at the end of the preceding century. It had become a case of "no new fundamental particle, no Nobel Prize".

At the same time, however, journals began to be filled with articles proposing how a laser might be built, often with diagrams showing scant respect for the rules that a light ray had to obey when traversing an optical system. There is more than a suspicion that the rejection by *Physical Review Letters* of Maiman's article describing the first working laser may have been due to its being misread as "another God damn laser proposal" rather than the landmark account that it proved to be. Not that the excitement was quite over at that point. The ruby was a pulsed laser: we still needed a continuous-wave model. Thus the first gas laser. Then we needed a semiconductor laser, which produced another thrilling race (with a strong gallic input). Then a tunable laser. And so on.

With the successful operation of the first few lasers, the number of new laser transitions increased explosively. Gone was the excitement of wondering if the next meeting of researchers would include the announcement of a new type of laser. Soon it was a matter of how many new ones would appear, then merely a listing of the next hundred or two, most of which were doomed never to be of any further interest.

In *The Laser in America*, Bromberg deals with the period leading up to the laser's invention as well as the subsequent decade of developments. She traces the way in which a remarkably small number of possible lasers eventually won out, tempting one to speculate on whether the present scene would be

much different if by chance others had been chosen. Would the helium–neon laser still reign supreme as a general laboratory tool if, for example, comparable effort had gone into designing a laser better matched to the maximum of the eye's sensitivity? Some proposed lasers, including Charles Townes and Arthur Schawlow's original potassium laser, have never worked. Others worked often without being clearly understood and were eventually forgotten.

Bromberg concentrates mainly on the factual side of the events of the period, which were confined mostly to the United States. The physics involved is discussed with both clarity and accuracy. She shows brilliantly how the laser and its developments markedly influenced many areas of post-war American policies, affecting education, research planning, the relation between large and small companies and much more. The sad but not uncommon controversies over "who thought of it first?" are reported without comment. It might be said that these were irrelevant in view of the explicit statement of the idea in a paper by Fabrikant that predates all the other claims. The question is raised (not for the first time) of why, given that the fundamentals of stimulated emission were first described in 1917 and that results were explained in these terms in 1932, it took until the sixth decade of the century for the maser and laser to appear. I believe that few will agree with Dunskey's view that "the concept of stimulated emission was not fully understood in the earlier period". The same view was expressed in many early discussions for the concept of coherence. Although not understood by some participants of conferences, such concepts were quite clearly set out and discussed long before the laser era.

The book is a delightful read and will be enjoyed by anyone with a passing interest in the history of the laser. Even for those not fortunate enough to have been involved in the early work, it will evoke much of the excitement of a period unique in the discovery and development of this remarkable device, whose value is in no way diminished by its current use in the supermarket checkout. □

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■ The rapid progress of laser science is attested to by the publication of three new books: *Lasers in Chemistry* by David L. Andrews (2nd edn, Springer, £22 (pbk)); *Laser Interaction and Related Plasma Phenomena Vol. 9* edited by H. Hora and G. H. Miley (workshop proceedings) (Plenum, \$150); and *Optical Lasers and Amplifiers* edited by P. W. France (Blackie, £69). □

Tales of old

Thomas B. L. Kirkwood

The Biology of Life Span. By Leonid Gavrilov and Natalia Gavrilova. Harwood Academic: 1991. Pp.385. \$120, £62.

Evolutionary Biology of Aging. By Michael Rose. Oxford University Press: 1991. Pp.221. £28, \$35.

Biology of Aging. By Robert Arking. Prentice Hall: 1991. Pp.420. £51.35, \$66.25.

ONE of Aristotle's many claims to fame is as the great granddaddy of gerontological science. "It is not clear whether in animals or plants universally it is a single or diverse cause that makes some to be long-lived, others short-lived." Thus mused Aristotle some 23 centuries ago in his *De Longitudine et Brevitate Vitae*. It is still a good question today, highlighting the core issues with which gerontology has grappled for so long.

The Biology of Life Span is a spirited plea that for too long the quantitative analysis of life span has been done both badly and not enough. As an updated translation of a work originally published five years ago in Russian, it provides a window into the extensive Soviet literature on ageing. It is also an optimistic book, describing with evident pride the Soviet Ministry of Health's special scientific programme on "extension of life". The specific thrust of Gavrilov and Gavrilova's argument is that an under-exploited richness of information lies scattered in the literature on demography and biology, which if read the right way could have answered many key questions of gerontology "some thirty years" ago. An appendix gives references to good-quality, published life-tables for as many species as Gavrilov and Gavrilova could discover.

There is interesting material examining the secular trends in human mortality, particularly the changing differences in survival between women and men, and exploring comparative aspects of life-span distributions. Later, the authors press for using reliability theory as a model for ageing. This idea is not a bad one, although it has been around for some time and needs to be carried through into carefully designed experiments. I found a bit tedious the extended discussion on whether life-span distributions really fit the normal distribution, and whether a species such as our own actually has a maximum life span. A modest dose of biostatistical common sense could have cut this discussion short.

A welcome addition to the bookshelf is *Evolutionary Biology of Aging*, which forcefully champions the relevance of

evolutionary theory to all aspects of research on this subject. In the first half, Rose deals directly with evolutionary issues, from explaining the theory, through to describing experimental tests of the evolutionary theories and genetic mechanisms for the evolution of altered life span. Particularly interesting are the experiments of Rose and others with *Drosophila melanogaster*, where selection for late reproductive ability results in increased life span. The finding is in accordance with the 'antagonistic pleiotropy' theory, which comes principally from a landmark paper by George Williams that was published in 1957. The idea is that genes conferring a fitness-advantage early in life will be favoured even if they cause the deleterious effects of senescence later on. The second half of the book deals briefly with the comparative biology of ageing, organismal theories of ageing, and cellular and molecular theories. Some of this material is a bit dated.

Biology of Aging is aimed at those requiring an introduction to the field. The style is engaging and light, and the book covers the main subject areas fairly well. Arking leads off with a comparative approach, dwells in some detail on human senescence, dips into various theories on the mechanisms of ageing, and concludes with a synthetic view of

ageing as a "genetically determined, environmentally modulated, event-driven process". What this means, in simple English, is that Arking sensibly recognizes that the control of longevity is best understood as a blend between genetic factors, such as genes that programme the efficacy of cell maintenance systems like DNA repair, and stochastic or environmental factors, such as those causing the lesions needing to be repaired. On the negative side, the book draws rather heavily on a few key sources, is weak on life-history theory, and spells people's names wrong rather often. But the pluses well outweigh the minuses, and the book should encourage bright students to study ageing further.

One swallow does not make a spring. Aristotle wrote that, too, in his *Nicomachean Ethics*. Similarly, three good new books on ageing do not herald the end of gerontology's problems. They are, however, a healthy sign. Understanding the mechanisms of ageing is one of the greatest challenges confronting biological and medical research. A great deal of hard science and a good measure of interdisciplinary cooperation will be needed to crack it. □

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Eccentric behaviour?

David W. Hughes

Comet Halley: Investigations, Results, Interpretations. Vol. 1: Organization, Plasma, Gas. Vol. 2: Dust, Nucleus, Evolution. Edited by J. W. Mason. *Ellis Horwood*: 1990. Pp.295 (Vol. 1), 275 (Vol. 2). Each £62, \$105.50.

AFTER the 1910 apparition of Halley's comet, astronomers had to wait some twenty-one years before Nicholas T. Bobrovnikoff at the Lick Observatory produced a comprehensive review of the results. Things have speeded up and expanded since then, and the thirtieth recorded apparition of the comet in 1985–1986 saw more of the world's scientists turn their attention to this once-in-a-lifetime visitor than ever before. We must congratulate John Mason on getting into print within four years.

Mason has assembled 39 detailed review articles into two volumes by calling on the expertise of a host of the world's top cometary experts and space scientists. The two volumes are targeted at the professional cometary astronomer. Other astronomers, including amateurs, will still have to wait for a more digesti-

ble account of the changes in our understanding of comets brought about by our observations of Halley.

Between January and March 1986, Halley's comet passed inside the Earth's orbit. For over two years around this period, many of the world's great telescopes were turned towards the comet. The astronomers in 1910 were confined to the use of the narrow visual band of the electromagnetic spectrum; 1986 saw full use of the ultraviolet and the infrared parts. Advantage was also taken of the new technologies of the space-age, an armada of six spacecraft rushing past the comet as it moved through the descending node of its orbit. No longer were cometary observers compelled to flatten their quarry onto the plane of the sky. They could now, for a brief moment, add the third dimension. They could also escape the confines of Earth and move very much closer.

The main success of the enterprise was to achieve the first-ever imaging of the cometary nucleus. The model of the central, kilometer sized, dirty snowball as the fount of all cometary activity, placed on a firm foundation by Fred L. Whipple 35 years before, was transformed into a reality.

The new collection of reviews is divided into six sections. The first looks at the organization and coordination of the scientific effort, involving the coopera-

tion of the European, Soviet and Japanese space agencies and the setting up of the International Halley Watch. The second section concentrates on plasmas and the complicated interaction between the solar-wind plasma streaming away from the Sun and the cometary plasma escaping from the vicinity of the sublimating snowy nucleus. Many fine images of disconnection events in the plasma tail are shown and much is made of the influence of the cometary plasma on the interplanetary magnetic field. The third section deals with the gas emitted by the comet, its flux, composition and spatial distribution. Next comes a section on the dust, with chapters on tails, jet morphology, size distribution, meteoroid streams and composition. The fifth section considers the nucleus, its shape, roughness, activity, mass, surface temperature distribution and spin state. The final section investigates the orbital evolution of comet Halley over the last few millennia and its physical evolution during that time.

We are presented with a detailed overview of what was discovered about the comet during its last apparition. The two volumes are superbly produced, lavishly illustrated, well referenced and usefully indexed. But to my mind there are two important deficiencies. First, too little attention is drawn to our ignorance. For example, the first tantalizing glimpses of a cometary nucleus resulted in a resolution of only about 150 metres and we have no idea as to the macro-properties of the dust and snow on the active and inactive regions of the comet's surface. Also, the spacecraft, moving at 150,000 miles per hour, could quantify only the fragments of the emitted cometary molecules and dust particles, and our detailed knowledge of their composition is negligible. The mass of the nucleus is known to a factor of four at best, and its internal dust, snow and density structure is still a complete mystery.

Second, there is no mention of the relationship between Halley's comet and all the other comets. We have benefited greatly from a fleeting glimpse of Halley, but have no firm idea about how typical it is. We are forced to use it in the calibration and interpretation of other cometary events, but it might be in itself quite unusual: we just do not know. □

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Correction

Hormones edited by Etienne-Emile Beaulieu and Paul A. Kelly (reviewed in *Nature* **351**, 362; 30 May 1991) is distributed by Chapman and Hall in the United Kingdom, the Commonwealth, the United States and Canada (£47.50, \$62.50 (pbk)). Hermann has all other European rights. □

The case for the relativistic hot Big Bang cosmology

P. J. E. Peebles, D. N. Schramm, E. L. Turner & R. G. Kron

The relativistic hot Big Bang model for the expanding Universe has yielded a set of interpretations and successful predictions that substantially outnumber the elements used in devising the theory, with no well-established empirical contradictions. It is reasonable to conclude that this standard cosmology has developed into a mature and believable physical model.

COSMOLOGISTS grow used to explaining to their colleagues in other fields why the latest reports of the death of the Big Bang model for the expanding Universe are premature, to say the least. To the contrary, we explain, in the six decades since the formulation of the model, advances in observations and experiments have yielded a considerable body of evidence in support of the Big Bang and none that convincingly contradicts it.

Reports of the death of the Big Bang, in popular media and professional journals (for example refs 1, 2), often confuse the Big Bang model with free parameters such as the present mean mass density. For example, the large structures observed in the distribution of galaxies^{3,4} are one of the serious problems for the set of ideas known as the cold dark matter theory for galaxy formation⁵. But they would be a problem for the Big Bang model itself only if it were shown that there is no plausible way to account for these structures within the relativistic expanding world model. As we will explain, there is no shortage of ideas on how it might be done^{6,7}.

A healthy degree of scepticism is in order, for we are using a short list of sometimes indirect evidence to arrive at a grand conclusion, that the Universe expanded from a dense hot beginning. Arp *et al.*¹, and many others^{2,8-14}, have given their accounts of the sceptical view; while these authors make some good points we feel they miss the three essential ones. First, we will argue that the standard expanding world model does fit, and even predict, the observations remarkably well. Second, the invention of a feasible alternative world picture that takes account of the full suite of observational and experimental evidence would require a good deal more ingenuity now than in past decades, when the debate was less fettered by physical evidence: today there is no known feasible alternative cosmology. And third, there are indeed open puzzles, such as the origin of galaxies, but none that seems to contradict the standard world model within present levels of understanding.

We describe below what has become the standard model in cosmology, and then present some highlights of the now substantial range of evidence that most cosmologists believe convincingly establishes the model. We are presenting only a selection of ideas, and have attempted to indicate further topics in the references. We end with brief speculations on how the open puzzles and work in progress might affect future developments in this field.

The standard model

By the standard Big Bang cosmological model we mean a Universe that is expanding according to Hubble's law,

$$v = H_0 r \quad (1)$$

where v is the recession velocity of a galaxy at distance r . Superimposed on this velocity field are the random motions of the galaxies, typically $\sim 500 \text{ km s}^{-1}$. There are relativistic corrections when v is comparable to the velocity of light, c , that is, at

distances comparable to the present Hubble length,

$$L = \frac{c}{H_0} \sim 6,000/h_{50} \text{ Mpc} \quad (2)$$

where $1 \text{ Mpc} \approx 3 \times 10^6$ light years and h_{50} is the Hubble constant in units of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$.

The wavelength of radiation from distant objects is stretched towards the red; the redshift factor z is defined by the ratio of the wavelength λ_e at emission (as measured by an observer at rest at the source) to the presently observed wavelength, λ_o ,

$$1 + z = \frac{\lambda_o}{\lambda_e} \quad (3)$$

When v in equation (1) is small this can be considered a Doppler effect, but it is better to note merely that the expansion of the Universe stretches the wavelength by the same factor by which the Universe expands between emission and observation. It is customary to label epochs in the evolving Universe by their redshift z even when z is so large that nothing reaches us without absorption and reradiation; then one thinks of z entirely in terms of the expansion factor. Thus since baryons (protons and neutrons) are conserved (at redshifts of interest here) the mean number density of baryons at epoch z is

$$n(z) = n_0(1+z)^3 \quad (4)$$

where n_0 is the present value.

In the standard model, the Universe has expanded from a state dense and hot enough that matter had relaxed to statistical thermal equilibrium. This means space was (and is) filled with black-body radiation, the cosmic background radiation (CBR). As the Universe expands, the number of CBR photons per unit volume drops, as in equation (4), and the photon wavelengths are stretched by the expansion, as in equation (3). The CBR thus preserves a black-body spectrum with a temperature that decreases as the Universe expands. The measured present temperature is^{15,16}

$$T_0 = 2.736 \pm 0.017 \text{ K} \quad (5)$$

The temperature at the epoch with redshift z is

$$T(z) = T_0(1+z) \quad (6)$$

As will be described, equations (4) to (6) have been tested back to $z \approx 10^{10}$. They cannot of course, apply all the way to $z \rightarrow \infty$. Ideas on the corrections to these equations associated with the speculative physics of the very early Universe¹⁷ are of great research interest but go beyond the standard model we are discussing. In this connection we might note that the name, Big Bang, is unfortunate because it may be misunderstood as referring to an event at a singular start of expansion of the Universe. Whatever started the expansion, perhaps an inflationary epoch, perhaps something even more wild, is not intrinsic to the standard model. Also, any prediction on where the

Universe is heading, to recollapse, eternal expansion or something else, is part of the uncertain parameters within the standard model.

The model assumes the Universe is homogeneous and isotropic in the average over scales comparable to the Hubble length in equation (2), that is apart from 'small-scale' fluctuations, the Universe looks the same in any direction and would look the same viewed from any other galaxy. This agrees with the precise isotropy of the background radiation fields and the unique temperature of the CBR, and at less precision, with galaxy counts. A useful measure of the scale at which the Universe approaches homogeneity is that the r.m.s. fluctuation in counts of galaxies found within a randomly placed sphere of radius r is^{4,18,19},

$$\delta N/N \approx 0.5 \quad \text{for} \quad r = 40h_{50}^{-1} \text{ Mpc} \quad (7)$$

and decreases as r increases.

The linear redshift-distance relation in equation (1) has the property that an observer on any galaxy sees the same recession law. This connection between homogeneity and Hubble's law was the first success of the expanding world model. The considerable subsequent improvements in the tests of homogeneity and Hubble's law (for example, refs 18–20, and Fig. 14 in ref. 21) over what was known when the relation was discovered must be counted as an impressive success for the standard model.

Finally, the dynamics of the expansion of the standard model are described by Einstein's general relativity theory. There is a rich and growing set of tests this theory²², but the main justification for its application to cosmology lies in its successes, as discussed below.

Cosmic background radiation

In the standard model, the Universe is filled with a uniform sea of thermal black-body radiation which was produced in the very early Universe and cooled by the expansion of the Universe. As indicated in Fig. 1, there is an isotropic radiation background detected at wavelengths $\sim 500 \mu\text{m}$ to $\sim 30 \text{ cm}$ with these properties. The boxes in the figure show the COBE satellite measurements, and the solid curve is the best-fit thermal Planck spectrum¹⁵. The boxes represent statistically independent measurements, and the box height is a nominal error bar, at 1% of the peak value of the brightness, which takes account of the uncertainty in a preliminary analysis of the calibration. The small scatter shows that these box heights are conservative. As it happened, a rocket measurement¹⁶ just a few weeks after the launch of COBE gave very similar results. Ground-based measurements at longer wavelengths are summarized by Wilkinson²³. All show that the CBR spectrum is very close to a thermal Planck function.

As matter has been hotter than the CBR for a long time, the CBR spectrum could not be exactly thermal. The fact that the energy density of the CBR is some nine orders of magnitude larger than the heat capacity of matter (at redshifts $z \lesssim 10^9$) makes it very difficult, however, to think of processes within the standard model that could appreciably perturb the spectrum. Practical evidence of this is to be found in the proceedings of cosmology conferences²⁴ held just before the recent spectrum measurement, when it was thought that the CBR temperature at wavelengths shorter than the peak might be $\sim 10\%$ higher than in equation (5). Heavy theoretical efforts failed to yield a credible theory that could fit the excess and the other available constraints. That is, given that the CBR effective temperature is about that in equation (5), the standard model says the spectrum has to be very close to thermal, as observed.

The thermal spectrum of the CBR is by now so familiar that we may forget just how specific is the prediction of the standard model, and how notable its experimental success. With just one adjustable parameter (the temperature in equation (5)), this prediction fits the sequence of independent measurements in Fig. 1 and the longer wavelength data. Any proposal of an

alternative to the standard model must face a substantial challenge in accounting for these measurements, as is shown in an example below for the steady-state theory.

Light element abundances and neutrino counting

In the standard model, the Universe cools as it expands. The expansion traces back in time to epochs when the Universe was hot and dense enough to drive thermonuclear reactions that changed the relative abundances of the chemical elements. The values of the abundances left over from this hot epoch depend on the cosmological parameters, but in a reasonably simple way. Knowing the present temperature, T_0 , (refs 15, 16), and assuming a value for the present matter density, we have fixed the thermal history of the Universe, including the density, temperature, and expansion rate through the nucleosynthesis epoch. In the standard computation (where matter is uniformly distributed and the lepton numbers are comparable to the baryon number), this is sufficient to fix the final abundances of the light elements, giving a remarkably good and detailed fit to the data^{25–30}.

The elements of cosmology and physics that go into the computation need not be discussed here in any detail, but it is worth emphasizing the immense extrapolation of the expansion of the Universe back in time, to an epoch when the CBR temperature was some ten orders of magnitude greater than it is now, and the number density of baryons 30 orders of magnitude larger. It is striking that this extrapolation yields a very successful prediction.

Figure 2 shows the abundances produced in the standard model. The free parameter, the present matter density, is expressed in two ways, as the baryon-to-photon ratio, η , or, equivalently, given the measured present CBR temperature, the fraction of the critical density (the density required to make the Universe's gravitational binding energy equal to its expansion kinetic energy) in baryons, Ω_b .

The vertical band in Fig. 2 is the range of allowed values of η that are simultaneously consistent with the observed light element abundances of ^4He , ^2H , ^3He and ^7Li extrapolated to their primordial values unassociated with any heavier elements. In particular, as reviewed in refs 27–30, the primordial mass fraction for ^4He is 0.23 ± 0.01 and present abundance ratios are $^2\text{H}/\text{H} \approx 2 \times 10^{-5}$ and $^3\text{He}/\text{H} \approx 2 \times 10^{-5}$. As significant amounts of ^2H cannot be produced in known noncosmological process³¹, only destroyed, the present abundance of ^2H puts an upper limit on the baryon density. Conversely, ^3He is made in stars, and as the bulk of the excess cosmological ^2H over the present value burns to ^3He in stars, the sum of ^2H plus ^3He provides a lower bound on the baryon density³¹. The allowed range of baryon density that is consistent with these bounds requires ^7Li to be near the minimum in its production curve (as shown in Fig. 2). Measurements^{32–34} giving $^7\text{Li}/\text{H} \approx 10^{-10}$ in the primitive (population II) stars further substantiate these arguments. Thus, the light elements with abundances ranging from $\sim 24\%$ to one part in 10^{10} of hydrogen all fit with the cosmological predictions, with the one adjustable parameter giving baryon density

$$\Omega_b = (0.06 \pm 0.02) h_{50}^{-2} \quad (8)$$

Recent attempts to escape this conclusion by introducing an inhomogeneous baryon distribution at the nucleosynthesis epoch (as might be caused by a first-order quark-hadron phase transition earlier still) have ended up³⁵ reaching essentially the same constraint on Ω_b as in the standard model.

Added to the impressive agreement of the abundances has been the measurement³⁶ using high-energy colliders of the number of neutrino families, $N_\nu = 2.98 \pm 0.06$. Nucleosynthesis arguments^{25,37} show that the cosmological ^4He abundance is quantitatively related to N_ν . The current parameter values^{28–30} yield the cosmological prediction $N_\nu \approx 3.3$, which specifically rules out any light neutrinos beyond e , μ and τ , and is consistent with the collider measurements. Thus accelerators as well as

telescopes test the standard model.

Finally, the predicted baryon density in equation (8) is within the range of estimates of the mean mass density in and around the bright parts of galaxies, including the dark massive haloes associated with galaxies as implied by their rotation curves^{28,30}. A more precise test of this point awaits identification of the nature of the halo dark matter detected through its gravitational effect in the outer parts of galaxies¹⁷.

To summarize, the standard model makes specific and successful predictions for light element abundances and N_{ν} . Any proposed alternative cosmology must face the particularly difficult questions of what (other than the Big Bang) could have produced the observed abundance of deuterium, an isotope readily destroyed in stars and not easily produced³¹, and the remarkably uniform abundance of helium in galaxies³⁰. This test supports the standard cosmological model back to the redshift of last equilibration of the neutron to proton ratio, $z \approx 10^{10}$.

The evolution of galaxies and quasars

Because of the light travel time, galaxies observed at great distances are seen as they were when they and the Universe were young. In the standard model the Universe was half its present age at a redshift in the range $z \approx 0.6$ –1, depending on the mean mass density. As modern observational techniques allow us to study objects at redshifts in and well above this range, there should be considerable observable evolution in the properties of the galaxies. This basic prediction of the standard model has been clearly established for galaxies and quasars.

The counts of galaxies as functions of redshift or brightness in the sky depend on the details of the geometry and evolution of the Universe, and also on how the galaxies are evolving; if younger galaxies were more luminous, more of them would be counted at a given brightness. There are observed to be more very distant galaxies (that is, those counted to very low brightnesses on the sky) than would be expected for nonevolving galaxies in the standard model (although the numbers are also roughly in line with the steady-state model to be discussed below)^{19,36}. In most cases the observed image parameters are consistent with mild evolution from normal galaxies³⁸. The deepest spectroscopic surveys^{39,40} show no evidence for galaxies with either remarkably high or low luminosity, nor do they reveal any population of sources that is altogether new. Rather, the observations for even the faintest images have been successfully modelled on the assumption that they are normal distant galaxies undergoing evolution of their stellar populations consistent with their younger ages in the standard model⁴¹. That is, it is believed that an evolutionary trend has been detected in the average population of galaxies at modest redshifts, $z \approx 0.5$.

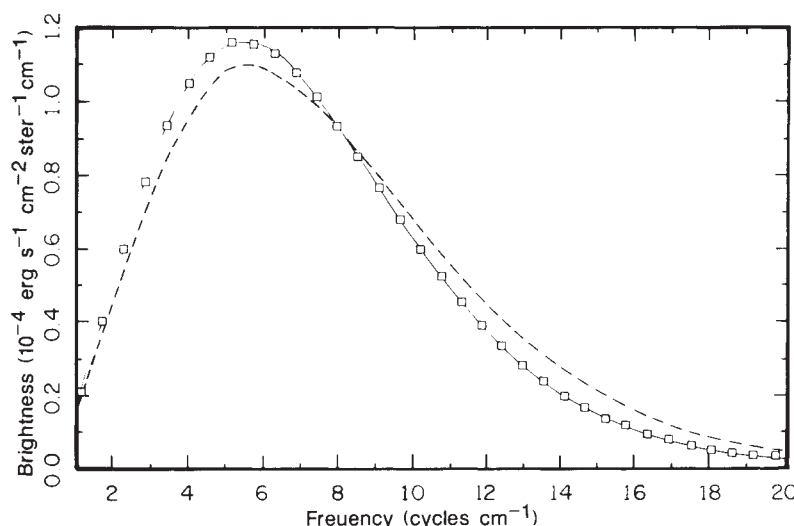
The situation for the small percentage of galaxies that are members of large clusters can be stated more simply. Compact rich clusters (those of high density and mass, containing many galaxies) are readily identified⁴² at redshifts up to $z \approx 1$, and have long been favoured cosmological landmarks. Some such clusters with redshifts $z \approx 0.3$ show a population of anomalously blue galaxies⁴³, and it has been demonstrated by spectroscopic studies that these blue galaxies are indeed in the high-redshift clusters (not foreground objects), and that they represent types of galaxies that do not occur in low-redshift clusters or even do not seem to occur at all at low redshifts⁴⁴. That is, galaxies in clusters show clear evidence of evolution (during the past few billion years in the standard model).

Galaxies that are strong radio sources can be identified to high redshifts and are thus also well suited to studies of evolution. To the largest observed galaxy redshifts, $z \approx 3.5$, the redshift appears to be a good measure of distance, judging from angular sizes of radio images and infrared apparent brightness⁴⁵. The optical-infrared morphologies of radio galaxies show a distinct trend with redshift, becoming more elongated and more closely aligned with the major axis of the radio image for $z \approx 0.6$, and there is evidence that some of this extended light originates in stars⁴⁶. Identification of complete samples of radio sources to faint optical limits provides a direct measure of the redshift distribution for radio galaxies, confirming the strong evolution indicated by the source counts⁴⁷. No clear understanding of radio galaxy evolution can be claimed, but it is certain that their morphologies change distinctly between high redshifts and low.

Finally, if the redshifts of nonthermal extragalactic sources (quasars and active galactic nuclei) are taken to be of the same nature as ordinary galaxy redshifts and thus indicative of distance (an issue addressed below), they show strong cosmic evolution. At redshifts $z \approx 2$ –2.5, when the Universe was 15–35% of its present age (depending on the mean mass density), high-luminosity quasars were at least a thousand times more numerous than they are at present^{48,49}. Moreover, the evidence is that the quasar population again decreases at still higher redshifts, extending out to the limits of observations at $z \approx 5$. That is, there is a rather well defined 'quasar epoch' in the history of the Universe⁵⁰. Lower-luminosity quasars and galaxies showing quasar-like activity in their nuclei are visible only out to $z \approx 1$, but they also show clear population evolution over this more recent period⁵¹.

Our conclusion is that discrete objects show distinct evidence of cosmic evolution. Ordinary galaxies, which have been studied only to fairly small redshifts (corresponding to relatively recent periods in the Universe's history), show relatively mild changes, while increasingly more prominent objects visible to higher

FIG. 1 Spectrum of the cosmic microwave background from COBE¹⁵. The boxes are statistically independent measurements. The solid line is the best fit for the thermal spectrum predicted by the standard model. The dashed line shows the predicted spectrum in a steady-state cosmology in which the optical depth in dust reaches unity at redshift $z \approx 0.3$.



redshifts (earlier epochs) show increasingly striking and extreme evolution. This is a qualitative result, as opposed to the quantitative tests discussed previously, but it is equally important as a test and success for the standard model.

Quasar distances and lensing

In the standard model, the expansion of the Universe shifts the light from a distant object towards the red. This cosmological redshift increases with increasing distance, so we expect high-redshift objects to lie behind those of low redshift. It is sometimes argued that certain classes of extragalactic objects, quasars in particular, violate this relation^{1,8}. If this were true, it would not necessarily rule out the standard model, which does not exclude other sorts of physical redshifts (indeed some, such as gravitational redshifts, are known to exist), but it certainly would raise the possibility of confusion or systematic error in the vital interpretation of galaxy redshifts as resulting from the expansion of the universe, so it is important to consider the question. It has a long history^{8,52,53}, only some highlights of which will be mentioned here.

The most common argument against the cosmological interpretation of quasar redshifts is based on apparent associations in the sky between high-redshift quasars and low-redshift galaxies. For example, Arp *et al.*¹ point out that of ~4,500 quasars so far identified⁵⁴, there are about 20 cases in which a low-redshift galaxy brighter than 15 mag appears within 2 arc-min of a high-redshift quasar, whereas less than two such cases would be expected if the quasars and galaxies were unrelated. Although this sounds compelling, it must be understood that the list of 4,500 includes all quasars found in any way, from systematic surveys to pure serendipity. If only a small fraction (≈ 0.005) of known quasars were discovered simply because they lie near bright galaxies, for example in images taken to study the galaxy, then the effect is entirely explained by a tiny selection bias. Such doubts concerning *a posteriori* hypotheses, subjective selection effects and lack of rigorous control surveys have left us and, we believe, most astronomers, unpersuaded by the case for large noncosmological redshifts. Nevertheless, there certainly are a few cases where the suggestion of physical association between low- and high-redshift objects is striking enough to warrant serious attention and further study. Amongst these, the NGC3067-3C232 system is perhaps the most notable⁵⁵.

These indications of associations between objects with very different redshifts are well worth following up, for if real they would provide striking evidence of new physics.

Where there is fairly direct and complete evidence of associations, it points not to new physics but to the standard cosmological interpretation of quasar redshifts. We review only the most recently studied and striking result, the gravitational lensing of quasars by foreground galaxies. (Not discussed here are several other lines of evidence for the standard interpretation.) Quasars at low redshifts, $z \approx 0.5$, are close enough on the cosmological interpretation that galaxies associated with the quasar should be visible. Low redshift quasars indeed have fuzz comparable to what would be expected from light from a normal host galaxy⁵⁶, and they have normal-looking companion galaxies in about the abundance observed for radio galaxies^{49,53}. Spectra of high-redshift quasars on lines of sight that happen to pass near low-redshift galaxies are observed to have absorption features, showing the quasars really are behind the galaxies⁵³. And the statistical properties of quasar absorption lines are independent of the line of sight to the quasar, indicating the lines arise from intervening intergalactic material⁵⁷.)

The lensing of a distant high-redshift quasar by a low-redshift galaxy or cluster of galaxies occurs when the two are closely enough aligned in the sky that the gravitational deflection of light rays by the mass in the foreground galaxy 'lens' is large enough to produce multiple images of the background quasar⁵⁸. Identification of a lensed quasar requires evidence that the quasar images are indeed multiple views of the same object. In such cases the masses known to be associated with galaxies or clusters of galaxies would produce multiple images with angular separations like those observed, provided the quasars lie far behind the lensing galaxies (so the bending angles required for the lensing are those expected for reasonable galaxy masses). That is, the lensing phenomena give us examples where the high-redshift quasar certainly is well behind the low-redshift galaxy.

In the few best instances, the case for a gravitational lens model of specific systems is detailed, extensive and compelling. These include the quasar Q0957+561, in which high-angular-resolution maps produced by very-long-baseline interferometry of the two source images show a mirror symmetry⁵⁹ predicted by the lens model, and the two images have identical flux variations (in the optical and radio) with a time delay consistent with the lensing geometry^{60,61}. In an 'Einstein ring' lens, the alignment of quasar and galaxy is so close and the mass distribution in the lensing galaxy close enough to axisymmetric that the galaxy images a region in the quasar as a ring centred on the galaxy. This is observed in the quasar MG1654+1346, where a galaxy at redshift 0.254 produces a classic Einstein ring lens image⁶² of the radio lobe of a quasar of redshift 1.74. A third example is the system Q2237+0305, for which a lens model fits a remarkably detailed set of observations.

The object Q2237+0305 has been offered as an example of evidence for noncosmological quasar redshifts¹, because of the small probability of such a precise alignment of a quasar with a low-redshift galaxy: probabilities ranging from $\sim 2 \times 10^{-6}$ to $\sim 9 \times 10^{-3}$ have been quoted^{63,64}. One has to be cautious of such statistics, for a specific real event may be exceedingly unlikely when compared with all the things that might have happened. If nevertheless one accepts that such an alignment exists, then the system matches the expected lensing to an extraordinary degree. Although Arp *et al.*¹ characterize its morphology as a 'blobby' QSO, high-resolution images obtained from the ground and more recently from the Hubble Space Telescope (Fig. 3), show four distinct unresolved images superimposed on an ordinary barred spiral galaxy. All four of these images show apparently identical emission-line spectra⁶⁶, and their separations are those expected given a conventional dynamical mass for the nucleus of the observed galaxy.⁶⁷ Moreover, the relative positions of the galactic nucleus and the four images are just

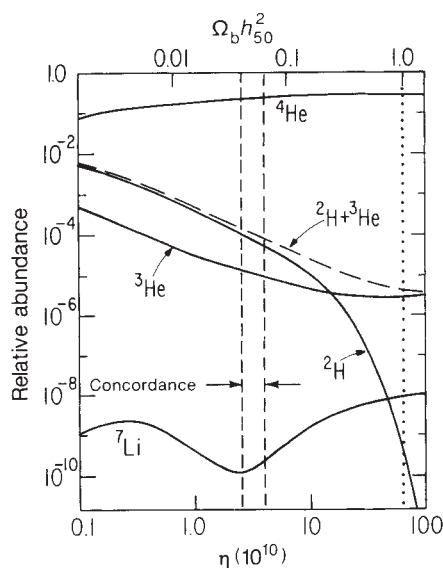


FIG. 2 Abundances predicted in the homogeneous standard model (Big Bang nucleosynthesis) as functions of the baryon to photon ratio, η , or, equivalently, the fraction of the critical density, Ω_b (based on ref. 26.) Relative abundances are by number, except for ^4He , which is given as mass fraction.

those predicted if it is assumed the mass that causes the lens is distributed like the observed starlight in the galaxy's nucleus⁶⁷. Finally, this lens model predicts independent flux variations in the four images because of 'micro-lensing' events in which a star in the lensing galaxy passes just across the path of one of the lines of sight to the quasar. This has now been observed⁶⁷⁻⁷⁰.

If this system were not a lens but four separate quasars physically associated with the galaxy¹, the nearly perfect resemblance to the lens system which would have resulted from a single quasar at its cosmological redshift distance behind the galaxy would be an extraordinary coincidence.

There is a substantial number of less dramatic instances which are plausibly understood as quasar lensing by galaxies⁷¹, and the frequency of lens candidates among quasar samples is consistent with that expected within the standard cosmology^{72,73}, if all observed quasars are at their inferred redshift distances.

To summarize, there is strong observational evidence for the gravitational lensing of quasars by foreground galaxies. Well before quasars were known it was predicted that, within the standard cosmological model, low-redshift galaxies would lens high-redshift objects⁷⁴⁻⁷⁶. If lensing were not observed in quasars, it would be a serious problem for the standard model. By the same token the positive observation is an important success. In particular, lensing gives us examples where high-redshift quasars manifestly are far behind low-redshift galaxies along nearly the same line of sight, consistent with the cosmological interpretation of the redshift.

The timescale problem

Reports of the death of the Big Bang often confuse the standard hot expanding universe model outlined in equations (1) to (6) with some specific version or extension. In particular, the Einstein-de Sitter model (in which space curvature and Einstein's cosmological constant Λ have a negligible influence on the expansion rate compared with the mean mass density) is attractive because it is particularly simple, and it is indicated by some interpretations of the physics of the very early Universe¹⁷. Two of the four authors of this report agree that the Einstein-de Sitter model is the strong sentimental favourite. But it also faces some potential problems. If it were observationally excluded we would learn that the Universe is more complicated

than some of us thought, but that is not an argument against the standard model.

Let us consider the timescale problem. The age t_0 of the Universe, calculated from a very high redshift to the present, and the Hubble parameter H_0 in equation (1), yield a dimensionless number $H_0 t_0$. In the Einstein-de Sitter model, $H_0 t_0 = 2/3$. If space curvature is significant but Λ is negligible, $H_0 t_0 < 1$. If Λ is significant, $H_0 t_0$ may exceed unity. Thus if H_0 and the ages of the oldest stars, which have to be no greater than t_0 , were known with enough accuracy, we would have an exceedingly valuable constraint on the cosmological parameters.

In a recent review, van den Bergh⁷⁷ finds

$$H_0 = 67 \pm 8 \text{ km s}^{-1} \text{ Mpc}^{-1} = 1/(15 \pm 2 \text{ Gyr}) \quad (9)$$

The main uncertainties in this very difficult measurement are systematic errors; other recent studies have yielded values in the range $H_0^{-1} \approx 10\text{--}20$ Gyr. The oldest reliably established ages of globular star clusters, based on stellar evolution theory, are $t_{GC} = 15 \pm 3$ Gyr (refs 78-80). Radioactive decay ages give $t_N > 10$ Gyr, independent of galactic evolutionary models, and when combined with evolution models indicate $t_N \approx 15 \pm 4$ Gyr (ref. 78).

It is impressive that the products $H_0 t_{GC}$ and $H_0 t_N$ are of the order of unity, for this unites the astronomical measurement of H_0 with the physics and astrophysics of stellar evolution and of the production and decay of the heavy elements. It is also interesting that the Einstein-de Sitter case, $H_0 t_0 = 2/3$, is within the realistic ranges of uncertainty quoted. Some astronomers are starting to suspect that $H_0 t_0$ will turn out to be larger than $2/3$. This 'timescale problem' is very relevant to the Einstein-de Sitter model universe, but easily resolved in the standard model: it would say that the mean mass density is lower than the Einstein-de Sitter case. The possible result $H_0 t_0 > 1$ would be particularly interesting, indicating a positive cosmological constant, Λ (refs 81, 82). But all of this must await improved accuracy in two extremely difficult measurements, of Hubble's constant and the ages of the oldest objects.

Origin of galaxies

Like the Einstein-de Sitter model, the cold dark matter (CDM) theory for galaxy formation⁵ is a particularly attractive special case that is in some observational trouble. If this case should

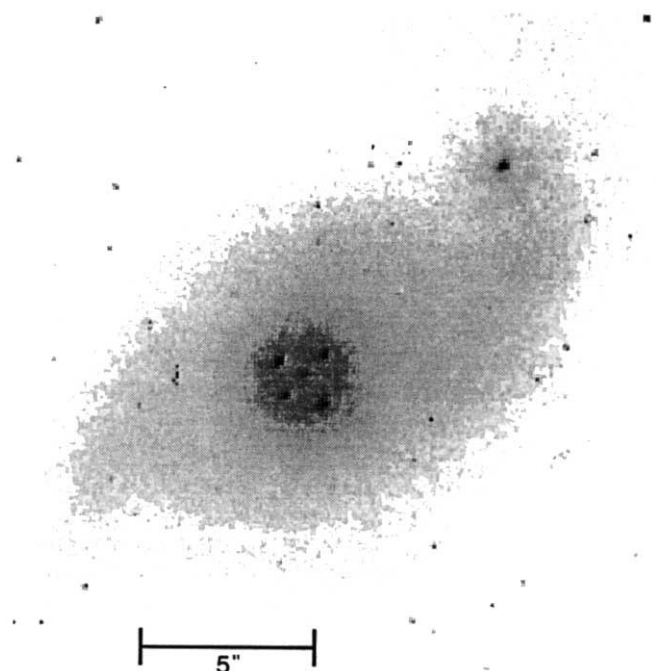


FIG. 3 This F702W (near infrared) band image of Q2237 +0305 obtained with the Hubble Space Telescope shows four distinct quasistellar images of the quasar at redshift 1.695 (the source). The ordinary barred spiral galaxy at redshift 0.0394 (the lens) is seen as the diffuse light centred on the four quasar images. (Photograph courtesy of NASA. Image taken with the JPL Wide Field Camera.)

be ruled out it would be a blow to simplicity, but not to the standard cosmological model.

The CDM model assumes (1) the seeds for galaxy formation come from small primordial gaussian density fluctuations with equal amplitude on all scales, and (2) the nonbaryonic dark matter necessary to get from $\Omega_b \leq 0.1$ to $\Omega_{\text{total}} = 1$ was slow moving (cold) at the time of galaxy formation.

Whatever the fate of the CDM model, it will be remembered as a working demonstration that matter in an expanding universe can assemble by gravity into objects not unlike galaxies with a roughly acceptable space distribution. The problems for CDM come not from the concept of dark matter *per se*, but rather from the seeds, which underproduce structure on the scale of clusters of galaxies or larger, assemble galaxies uncomfortably late, and give no credible explanation for the observation that the voids in the galaxy distribution, where most of the matter is supposed to be, appear to be so empty. The question naturally arises, if the CDM model cannot be saved, can we find a reasonable replacement? Given the remarkable isotropy of the CBR, it is fair to ask what level of isotropy would eliminate all models of structure formation in the standard Big Bang framework.

The CDM prediction for the CBR isotropy is not yet in serious trouble⁸³. Gaussian seeds with CDM require a minimum quadrupole anisotropy $\sim 7 \times 10^{-6}$, assuming that 100-Mpc structures exist today. This limit should be achievable by COBE and will definitely test the model. If the CDM model is eliminated, at least three alternatives remain viable. First, one can drop the assumption of gaussian density fluctuations: in models where structure is seeded by textures⁸⁴ or cosmic strings⁸⁵ the *primaeva* fluctuations are strongly nongaussian. That is a great help in making galaxies form earlier, and not in the voids, without overproducing the fluctuations to which most CBR anisotropy measurements are sensitive. Second, traditional models for generating seeds, whether gaussian or topological (such as textures), assume the seeds are generated in some early vacuum phase transition, long before the decoupling of the matter and radiation, but if the phase transition or other nonlinear process that generates seeds happened after decoupling it could strongly perturb the mass distribution without appreciably affecting the CBR⁷. Each of these options would still predict $\delta T/T \geq 10^{-6}$ in order to yield the observed structures, but the anisotropy could be nongaussian. Third, the Universe may have a mass density appreciably lower than the Einstein-de Sitter case. Lowering the density increases the length at high redshift subtended by a given angle, which means the anisotropy measurements on large angular scales probe structure on scales much larger than those on which we have measurements to tell us what the structure ought to be. Anisotropy on scales of a few degrees or less is prey to the complications of astrophysics. The betting is that as anisotropy measurements improve to values of $\delta T/T$ less than a few parts in 10^5 , the signal will be dominated by discrete extragalactic radio sources, emission from our Galaxy and the perturbation by hot gas in clusters of galaxies. In a low-density universe it is easy to imagine⁶ that scattering in clouds of cool plasma at redshifts $z \approx 20$ –30 smooths the *primaeva* fluctuations in the CBR on comoving length scales of $\sim 1,000$ Mpc, an order of magnitude larger than the largest observed structures. The scattering would replace the intrinsic anisotropy with what is reinserted by the structures of the scattering clouds. Whether the effects can be untangled is a subject for future research.

Our feeling is that the main point of interest here is not the relative prospects for any specific *ab initio* model for galaxy formation, but rather that the fund of observational clues to what really happened is growing at a large and increasing rate⁸⁶. A clearer picture of structure formation should eventually emerge. If no anisotropies in the CBR are seen down to $\delta T/T < 10^{-6}$, then one may indeed question the basic cosmological picture.

Alternative cosmologies

The standard model passes all believable tests to date, but one can ask whether there is an alternative cosmology that can do as well or better. This is important not only because we should be alert to new ideas but also because it gives us an indication of how seriously to take the successes of the standard model: if alternative cosmologies also were consistent with the data it would suggest that available observations are inadequate as critical tests of world models. That is the case for some tests. For example, the linearity of Hubble's law in equation (1) follows from the homogeneity assumption and local Lorentz invariance, independent of any further elements of general relativity theory. We will argue, however, that no other cosmological model proposed so far fits the full suite of observational constraints.

The traditional alternative cosmology, the steady-state model^{9,10}, assumed a homogeneous expanding universe which is not evolving because matter is continually created so as to preserve a steady mean density. This model provides a useful foil for the discussion of the significance of the CBR spectrum measurements.

In the steady-state theory it would be reasonable to suppose radiation is created along with the continuous creation of baryons, but absurd to suppose the spectrum of the created radiation is just such that the integrated background, taking account of the varying redshifts of radiation created at different distances from us, adds up to a thermal form. To make an alternative theory for the spectrum of the CBR within the steady-state theory, let us postulate that the created energy is absorbed by intergalactic dust and reradiated by the dust with a thermal spectrum at constant temperature T . (This is a version of the picture proposed by Arp *et al.*¹) The optical depth for this process, measured in units of reciprocal light travel time, is κ . For simplicity we will suppose κ is independent of wavelength in the range of COBE satellite measurements in Fig. 1. Then in the classical steady-state model the integrated surface brightness (energy flux per steradian and unit frequency interval) due to the thermal emission of the dust is

$$i(\nu) = \int_0^\infty \kappa d\tau e^{-(3H_0 + \kappa)\tau} P(e^{H_0\tau}\nu) \quad (10)$$

Here τ is the lookback time, $t_0 - t$, the Hubble parameter H_0 is independent of time, and $P(\nu)$ is the thermal Planck spectrum at the constant dust temperature T . The absorption of the dust would reduce the observed flux density f_0 from a radio source at redshift z from the value f in the absence of the dust, by the relation

$$f_0 = f(1+z)^{-\kappa/H_0} \quad (11)$$

Equation (10) says that if $\kappa \gg H_0$ then $i(\nu)$ is close to a Planck spectrum. This corresponds to a case in which the Universe becomes black (opaque) at a distance corresponding to a low redshift, so the radiation we detect has not been appreciably affected by the redshift.

The dashed line in Fig. 1 shows the predicted spectrum with $H_0 = 0.3\kappa$ and the dust temperature adjusted to get the best fit to the data. Here the Universe reaches unit optical depth at redshift $z \approx 0.3$. The radiation coming from dust grains at redshift z has its temperature lowered by the factor $(1+z)^{-1}$ (equation (6)), so we see an admixture of temperatures, which add up to the distinctly nonthermal spectrum shown as the dashed line. This case manifestly is excluded by the observations.

With

$$\kappa/H_0 = 10 \quad (12)$$

the predicted spectrum from equation (10) is below the error boxes in Fig. 1 at wavenumbers 3–6 cm^{-1} , and above the error boxes at wavenumbers 11–16 cm^{-1} . As the error boxes are very conservative, this case also is excluded; still higher opacity is

needed. But according to equations (11) and (12) the radio flux from a source at redshift $z = 2$ would be attenuated by a factor of 10^5 . That certainly does not agree with the observation of high-redshift radio galaxies at wavelengths ~ 2 cm that show no indication of absorption extrinsic to the source⁸⁷.

The conclusion is that, even with the freedom to adjust the amount of intergalactic dust, the classical steady-state model cannot account for the distinctive thermal spectrum of the CBR: if the opacity of the dust is low enough to agree with the observations of radio galaxies at high redshifts then the theory predicts that the CBR is an admixture of thermal spectra at different redshifted temperatures, which is not observed. And of course this model leaves unexplained other observations discussed above: the systematics of light element abundances, and the cosmic evolution of extragalactic objects.

Hoyle has consistently emphasized that the perfect cosmological principle of the steady-state theory (which says that the Universe is invariant under translations in time as well as space) might apply only in the average over long times, just as the homogeneity condition in the standard model ignores local fluctuations in the mass distribution. Even before the discovery of the CBR, Hoyle and Narlikar⁸⁸ considered a model in which creation occurs in bursts between which the Universe evolves as in the standard model. If the bursts are dense, hot and rare enough the results can be indistinguishable from the standard model (and can be considered a forerunner of the inflation picture).

Another alternative is the cold Big Bang model, in which the CBR would be produced and thermalized by dust at some fairly recent epoch, labelled by redshift $z \approx z_1$. If $z_1 \geq 5$, which is beyond the most distant observed quasars, the dust would not violate the observed transparency of the Universe. Wright⁸⁹ showed that one can find physically realizable models for the dust (graphite or iron needles) and the source of radiation (neutron stars or black holes accreting much of the mass of the Universe and reradiating the energy at the theoretical Eddington upper limit) at $z_1 \approx 200$ that fitted the spectrum measurements then available. This model requires highly special conditions, and of course it fails to account for the light element abundances, but still it would be interesting to see whether the model could be arranged to fit the new spectrum measurements^{15,16}.

In Alfvén's¹¹ 'Plasma Universe', we are part of a finite cloud of material expanding into empty asymptotically flat space. If the CBR were present in the otherwise empty space into which the galaxies are moving, it would be an extreme coincidence that we are moving at the observed low velocity ($\sim 600 \text{ km s}^{-1}$) relative to the CBR²³, as most galaxies are moving away from us at relativistic speeds. In the version discussed by Lerner⁹⁰, the CBR is radiated from material in the expanding cloud. Here the abovementioned problem of making the opacity high enough to get a thermal spectrum without obscuring distant radio galaxies is exacerbated by the fact that the cloud would hardly be expected to be spherically symmetric to the degree required to account for the isotropy of the CBR²³ (isotropic to better than one part in 10^4 on angular scales from ~ 15 arcmin to 180°). The anisotropic expansion of the plasma cloud would cause anisotropic dimming of the CBR because of the redshift, in violation of the observations. In this model, rifts in the emitting clouds could permit observations of high-redshift radio galaxies, but that would imply extreme variability in the mean redshift of the CBR in different directions, causing strong anisotropy, again in violation of the observations.

The 'chronometric' theory of Segal¹², which gives a quadratic redshift-distance relation, would have been a useful spur to discussion if introduced in the late 1920s, when the linear relation was proposed on limited observational grounds. But the modern tests of the redshift distance relation^{20,21} convincingly rule out the chronometric relation. Discussions of this model have not even reached the stage of addressing the more

subtle problems of the CBR spectrum and the light element abundances.

Finally, in a pure fractal model, the galaxy distribution is a scale-invariant clustering hierarchy^{13,14}. On small scales, the observed galaxy distribution is well approximated⁹¹ as a fractal with dimension $D = 1.23 \pm 0.04$. As Mandelbrot's¹³ examples show, however, a pure fractal (with a single dimension) has the same appearance under a change of scale. That is, in a pure fractal universe the galaxy distribution would be as clumpy in deep samples as in shallow ones, quite contrary to the observations^{4,19,91}.

The study of alternative cosmologies is a natural and wise thing, for the standard model was proposed in the late 1920s on the slightest of theoretical grounds (Mach's principle) and observational evidence (marginally significant indications of large-scale uniformity in the galaxy distribution, and of a linear redshift-distance relation for nearby galaxies)⁹². It would have been natural to expect that this naive model would have to be heavily modified or even abandoned as more data became available. So far, however, that has not happened. Though some of the alternate cosmologies are elegant and theoretically compelling, it is the original relativistic expanding universe that so far best fits the observations.

Evaluation and opportunity

How might our subject be affected by results of research to come? If favoured ideas fail, what will we most readily abandon?

It is easy to think of results that would severely weaken or even eliminate the credibility of the standard cosmological model. This is a 'good thing', for theory that is not falsifiable is not interesting. If the galaxy distribution had been observed to follow a pure scale-invariant fractal, a possibility that would have been difficult to exclude in the late 1920s, the closely thermal spectrum and isotropy of the CBR in this highly inhomogeneous Universe would have been a deep puzzle. It will be very embarrassing if deep galaxy surveys now in progress show that fluctuations in the space density of galaxies averaged over scales of the order of the Hubble length in equation (2) exceed the limit $\delta N/N \approx 1 \times 10^{-4}$ allowed for mass fluctuations by the isotropy of the cosmic background radiation. Whatever the baryon distribution at light element nucleosynthesis, the match of theory to observation indicates that the primordial ^4He mass fraction should be near 24%. The discovery of an extragalactic object with a well established mass fraction in ^4He of, say, 0.15 would be another serious, if not fatal, problem. The same would have been true if experiments at the Large Electron Positron collider (LEP) had found $N_\nu = 5$ light neutrino families, instead of the predicted and observed values close to three. Astronomers could have discovered a class of distant objects with large blue shifts. That would have shown that the fundamental kinematics of the Big Bang model are seriously incomplete. And, for a final example, the discovery of a star cluster with a stellar evolutionary age of, say, 10^{11} years would put the product $H_0 t_0$ well outside the range allowed by the uncertainty in the cosmological parameters.

What do we do about inflation? The division of opinion among the authors of this review may be a reasonably close reflection of opinion in the community. Half of us consider inflation attractive, and would deeply regret its loss if it were somehow shown to be wrong. The other half wonder why we care so deeply about a theory that makes so few definite testable predictions. Perhaps quantum gravity (or string theory?) will one day find an alternative to inflation for setting the cosmological initial conditions. But pending further growth of understanding of physics under extreme conditions, we live with inflation, as one of the open puzzles.

What do we do about the Einstein-de Sitter model? It would have to be abandoned if the Hubble constant were shown to be $H_0 > 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$. If observations revealed objects

unambiguously older than 17 Gyr, it would drive us to a cosmological constant. Many of us would regret both steps as somehow inelegant. Some would take comfort in the fact that estimates of the mean mass density on scales of clusters and galaxies have been pointing in the same direction. All would conclude that the Universe is even more complicated than some of us hoped. But we emphasize once again, this all is within the standard expanding relativistic world picture.

What do we do about galaxy formation? The remarkable isotropy of the CBR cuts two ways. It is strong evidence for

the large-scale homogeneity of the Universe, but it considerably limits options for the departures from homogeneity that must be present in galaxies and the large-scale clustering of galaxies. The issue may yet end in a theory that violates no observation, yet is too complicated to be convincing. But this is a vague worry about simple preliminary attempts at a complex problem. Observations at last are revealing the nature of objects at redshifts greater than unity, when our model says the Universe was young, thus giving direct clues to what really happened⁸⁶. □

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The B-cell antigen receptor of the five immunoglobulin classes

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Several proteins associate with surface IgM to form the antigen receptor. We show that just two, the α and β associated chains, are sufficient to reconstitute an IgM surface receptor in fibroblasts. Contrary to expectation, a common α chain associates with all five immunoglobulin classes. We propose that B-cell antigen receptors consist of a common α/β heterodimer associated with each immunoglobulin class. But the classes differ both in the glycosylation of their associated α chain and in their dependence on α/β for surface transport.

SURFACE immunoglobulin in B lymphocytes acts as a receptor for antigen¹ and is implicated both in B-cell activation and in the induction of tolerance^{2,3}. The endocytosis of surface immunoglobulin allows the internalization of antigen and its subsequent processing and presentation to T cells⁴. The cross-linking of surface immunoglobulin by antigen leads to transmembrane signalling¹. Membrane immunoglobulin has only a short cytoplasmic tail and exists in the cell surface noncovalently associated with several proteins. Those proteins associated with surface IgM have been most extensively studied⁵⁻¹², though only two polypeptides have been well characterized^{7,8}, the IgM- α and Ig- β , which are encoded by the *mb-1* and *B29* genes, respectively^{7,8,13,14}; the two polypeptides form a disulphide-linked heterodimer¹⁵. In the absence of IgM- α , membrane IgM is retained in the endoplasmic reticulum and degraded intracellularly^{16,17}. Mutagenesis studies¹⁸ suggest that the IgM-associated proteins form a sheath around the μ transmembrane segment as it is the hydrophilic amino-acid residues in this segment which are responsible for this intracellular retention.

The molecular mechanism of transmembrane signalling by surface immunoglobulin is little understood. The IgM-associated proteins are probably involved as receptor crosslinking results in the rapid phosphorylation of IgM- α and Ig- β on tyrosine residues^{9,19}. There are indications that IgM and IgD can transduce different signals²⁰⁻²³ although the functional difference between these two isotypes is unclear. Membrane IgD may be associated with an IgD- α /Ig- β heterodimer composed of a β subunit which is identical to that associated with IgM and an α subunit which is larger than IgM- α . This is supported by immunoprecipitation data and transfection experiments which indicate that IgM and IgD differ in their requirements for proteins which mediate cell-surface transport^{10,24}. This suggests that each immunoglobulin isotype may be associated with a heterodimer composed of a common Ig- β subunit and an isotype-specific α subunit. This work was done to define more clearly the antigen receptor of the IgM class and compare it to the receptors of the other isotypes.

Reconstitution of a surface IgM complex

Because a coreceptor is required to facilitate transport of membrane IgM to the cell surface, we identified its components in reconstitution experiments in nonlymphoid cells. We used μ_m , λ , *mb-1* and *B29* expression units to establish stable transfectants of mouse NIH 3T3 fibroblasts expressing $\mu_m + \lambda$ chains together with IgM- α chains alone, Ig- β alone or both IgM- α and Ig- β . Immunofluorescence (Fig. 1a, b) revealed that IgM was transported to the fibroblast surface only when both IgM- α and Ig- β chains were present. Moreover, the IgM- α /Ig- β heterodimer remained associated with the membrane IgM on the cell surface (Fig. 1c). Thus, no other lymphoid-specific protein is needed to potentiate IgM surface transport. The IgM- α /Ig- β heterodimer must mediate its effect through the C-terminal part of the μ_m because it can also potentiate surface transport of a CD8/ μ chimaeric protein (Fig. 1d), a molecule which is otherwise retained intracellularly¹⁸.

IgD- α is encoded by *mb-1*

To study the coreceptor associated with IgD, we introduced a plasmid that directs expression of the membrane form of an IgD, $\lambda 1$ antibody specific for the hapten 4-hydroxy-5-iodo-3-nitrophenacetyl (NIP) into the M12 mouse B-cell lymphoma (Fig. 2a). Surface-radiolabelled IgD from a digitonin lysate of the M12[δ_m, λ] transfectant was run on SDS-PAGE and compared with an M12[μ_m, λ] transfectant [Fig. 2b]. From comparison with other data^{10,24}, the diffuse bands of relative molecular mass of about 38,000 (M_r , 38K) that are common to the IgM and IgD complexes correspond to Ig- β , whereas the IgD-associated band of 34K (which is larger than the corresponding 32K IgM-associated band) has been designated IgD- α . This IgD- α subunit was purified from large-scale cultures of the M12[δ_m, λ] cells and fragments of the α polypeptide were generated by CNBr digestion. One of the peptides gave an identifiable sequence (Fig. 2c). Screening of the PIR database with this sequence gave only one good match, namely in the cytoplasmic tail of *mb-1* itself. This suggested that the α chain of the surface IgD complex is encoded by a gene which is identical or closely related to *mb-1*.

Screening of a genomic Southern blot at low stringency with a probe covering the cytoplasmic tail of *mb-1* did not reveal any bands other than those seen under high-stringency conditions (not shown). To test whether the *mb-1* product itself could associate with IgD, we transfected an *mb-1* transcription unit together with the δ_m gene into the J558L plasmacytoma; J558L transcribes both a λ light-chain gene and *B29* but expresses neither *mb-1* nor an immunoglobulin heavy-chain gene of its own. The transfectants stained brightly on the surface for IgD and the IgD was associated with a heterodimer indistinguishable from that detected in the IgD-expressing M12 transfectants (Fig. 3a). Although these observations strongly support the idea that the α chain of the IgD complex was encoded by *mb-1*, they do not directly prove it. We therefore resorted to gene tagging and cotransfected J558L with a plasmid encoding both δ_m and an *mb-1* expression unit in which the C-terminal end of *mb-1* has been mutated so as to add on a peptide²⁵ which can be detected using the monoclonal antibody, MYC1-9E10.2.

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Western blotting of the IgD purified from the J558L[δ_m ,*mb-1*^{tag}] transfectants (Fig. 3b) demonstrated that IgD associates with the tagged *mb-1* product. The different mobilities of the α chains of the IgM and IgD complexes are most probably accounted for by post-translational modifications. Indeed, after deglycosylation treatment, there is no difference in the mobilities of the α chains of the two complexes (Fig. 2b, right panel), consistent with previous results¹⁰.

Association of *mb-1* with IgG, IgA and IgE

Analysis on two-dimensional gels of surface immunoglobulin from M12 cells transfected with plasmids directing the

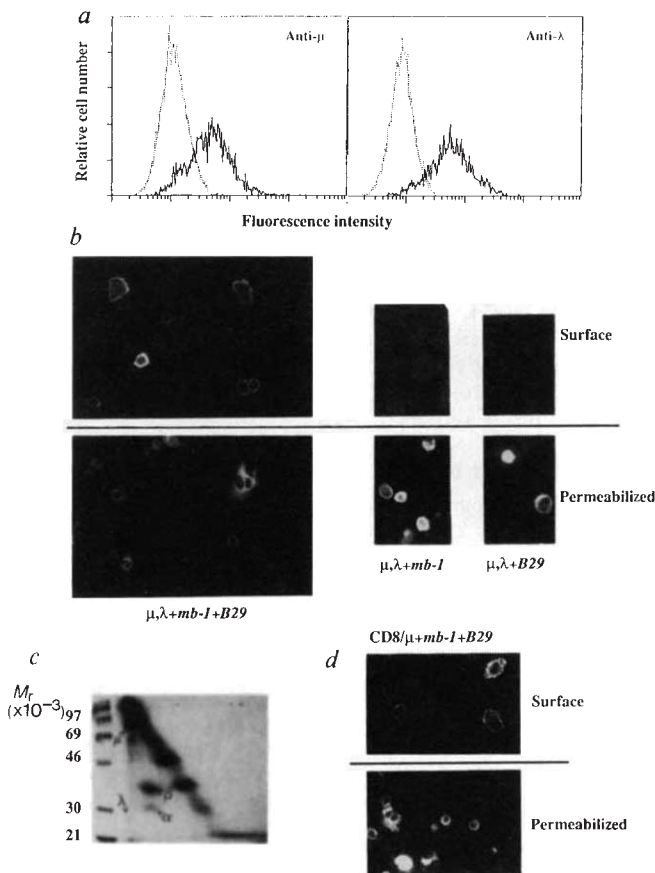


FIG. 1 The α/β heterodimer is sufficient to mediate transport of membrane IgM to the surface of nonlymphoid cells. **a**, FACS analysis of NIH3T3 cells stably cotransfected with a pSVgpt- μ_m , λ plasmid together with a pSVneo-*mb-1/B29* plasmid and then stained on the surface with FITC-conjugated anti- μ or anti- λ antisera (solid line). The dotted line shows staining of untransfected control cells. **b**, Immunofluorescence of the NIH3T3[μ_m , λ ,*mb-1*,*B29*], NIH3T3[μ_m , λ ,*mb-1*] and NIH3T3[μ_m , λ ,*B29*] cells stained with anti- μ with or without NP-40 permeabilization. **c**, Analysis by two-dimensional SDS-PAGE of radiolabelled surface IgM purified on hapten columns from digitonin-lysed NIH3T3[μ_m , λ ,*mb-1*,*B29*] cells. **d**, Immunofluorescence of stably transfected NIH3T3[CD8 μ ,*mb-1*,*B29*] cells stained with FITC-conjugated anti-CD8 (Ortho) with or without NP-40 permeabilization. **METHODS**. Plasmids directing expression exclusively of the membrane form of a mouse IgM, λ anti-NIP antibody¹⁶ or of a CD8/ μ chimaeric protein¹⁸ were introduced into cells by calcium phosphate coprecipitation (NIH3T3). The *mb-1* transcription unit was made by substituting the region between the *Nco*I and *Bam*HI sites of the human β -globin gene by an *mb-1* cDNA (generated by (PCR) polymerase chain reaction using an oligo-nucleotide spanning the AUG initiator codon and another spanning the termination codon). The *B29* transcription unit was similarly constructed by PCR from cDNA. Cells were surface-labelled with ¹²⁵I-labelled water-soluble Bolton Hunter reagent³⁰ and lysed in 1% digitonin. Labelled antibody was purified by binding onto NIP-caproic Sepharose and elution with free hapten³¹; samples were analysed on a two-dimensional gel (first dimension non-reducing, second dimension a reducing 10.6% SDS-PAGE).

expression of IgG2b or IgE revealed that both isotypes associated with a heterodimer analogous to that associated with IgM; furthermore, as in the case of IgM, this association is sensitive to Nonidet P-40 (NP-40; Fig. 4). To determine whether the *mb-1* product could associate with these immunoglobulin classes and whether it could potentiate their surface transport in plasmacytoma, we compared surface immunoglobulin expression in J558L cells transfected with either $\gamma 2b_m$, α_m or ϵ_m plasmids alone or with plasmids that also included the *mb-1* expression unit. As in the case of IgM, the *mb-1* product mediates the surface transport of both IgE and IgA (Fig. 5a). With IgG2b, however, there was surface transport in the absence of *mb-1* and analysis of surface IgG2b from radiolabelled J558L[$\gamma 2b_m$] cells on one- (Fig. 6b) and two-dimensional (not shown) gels did not show any detergent-sensitive association with a heterodimer. Nevertheless, a two-dimensional gel analysis (Fig. 5b) reveals that when *mb-1* and $\gamma 2b_m$ are coexpressed in J558L, then the surface IgG2b is associated with α/β . Thus, IgG2b differs from IgM in that it does not require *mb-1* for

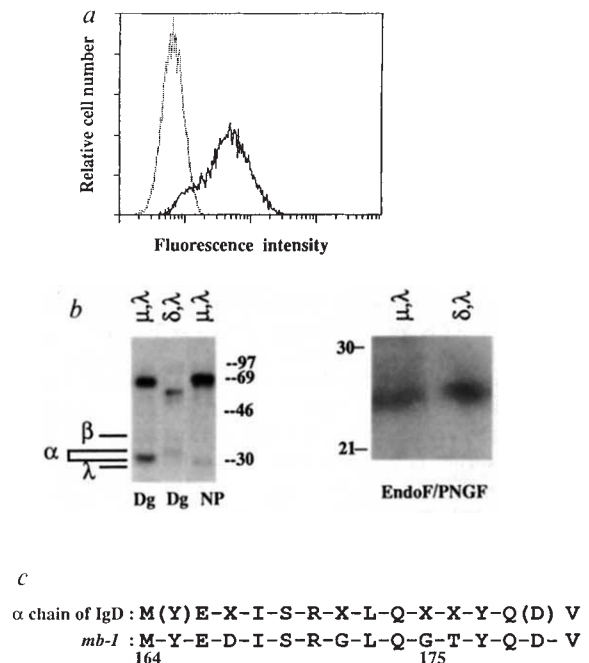


FIG. 2 Proteins associated with IgD in B cell lymphoma. **a**, FACS profiles of M12[δ_m , λ] (solid line) and of untransfected M12 (dotted line) stained with FITC-conjugated anti- δ antiserum. **b**, Comparison by SDS-PAGE of the proteins associated with purified surface IgM and surface IgD in M12[μ_m , λ] and M12[δ_m , λ]. Bands corresponding to λ light chain and to the α - and β -associated subunits are indicated. Surface immunoglobulin was purified on NIP-Sepharose from cells that had been surface radiolabelled and then lysed in the presence of either digitonin (Dg) or 1% NP-40 (NP). Binding of α/β to immunoglobulin is sensitive^{9,15,24} to NP-40. The right-hand panel shows the mobility in SDS-PAGE of the α bands associated with surface IgM and IgD in the M12 transfectants following deglycosylation. **c**, Comparison of the sequence of a CNBr fragment generated from the purified IgD-associated α protein with the corresponding sequence of *mb-1*. The depicted methionine residue is inferred (single-letter amino-acid code). Residues depicted in parentheses are tentative assignments whereas 'X' indicates a residue where no assignment could be made. **METHODS**. Deglycosylation of hapten-purified immunoglobulin samples was done for 18 h at 37 °C using endoglycosidase F and poly-N-glycosidase F (endoF/PNGF, Boehringer). The α bands could be identified by comparison of transfectants carrying tagged and untagged *mb-1* genes. For peptide sequencing, NIP-binding antibody from 2×10^{10} digitonin-lysed M12[δ_m , λ] cells was run on SDS-PAGE and the α band electroeluted, subjected to CNBr digestion and the resultant fragments purified by HPLC on a C8 reverse-phase column in an acetonitrile/trifluoroacetic acid gradient. Peptide sequence was determined using an Applied Biosystems automated gas-phase sequencer.

FIG. 3 The *mb-1* gene product associates with IgD. *a*, Analysis by two-dimensional SDS-PAGE of the radiolabelled surface IgD complex from J558L[δ_m ,*mb-1*]. *b*, Association of *mb-1*^{tag} with IgD by western blotting. Immunoglobulin purified on NIP-Sepharose from J558L[δ_m ,*mb-1*^{tag}] and J558L[$\gamma 2b_m$, *mb-1*] (control) cells was subjected to SDS-PAGE and western blotted with an anti-tag antibody.

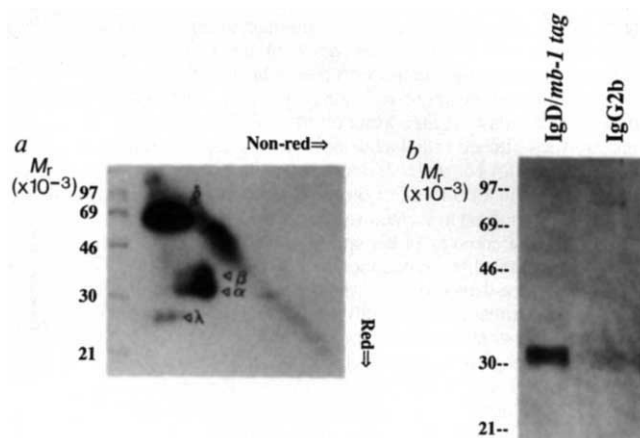
METHODS. Plasmids which direct the expression of the membrane form of mouse IgD and IgG2b were constructed from genomic DNA and for IgE and IgA by PCR to remove the secretory IgH polyadenylation sites and delete much of the intron separating the C_H exons from the membrane exons. A tagged derivative of *mb-1* was also made in which codons for the amino acids EQKLISEEDL (a sequence which is recognized by the monoclonal antibody²⁵ MYC1-9E10.2) were added at the *mb-1* C terminus. The *mb-1* and *mb-1*^{tag} transcription units were cloned into the pSVgpt- δ_m vector and stable transfectants were established by electroporation¹⁸. For western blot analysis, immunoglobulin purified on hapten columns from digitonin lysates was subjected to one-dimensional SDS-PAGE. Protein was transferred to nitrocellulose and reacted with the MYC1-9E10.2 antibody²⁵ (gift of R. Hawkins); bound antibody was detected using biotinylated anti-mouse κ antiserum followed by streptavidin/horseradish peroxidase complex (Amersham).

surface transport although it will associate with the α/β heterodimer when present.

Comparison of the α chains associated with IgM and IgD in the J558L transfectants reveals a difference in mobility similar to that detected in M12 (Figs 2*b* and 5*c*). The α chain of IgG2b is similar to that of IgM (Fig. 5*c*) and that of IgA is similar to IgD, whereas comparison of the IgD and IgE α -polypeptides reveals differences attributable to glycosylation (Fig. 5*c*). Comparison of the pattern of bands given in the two-dimensional gels by the IgG2b-associated proteins of the M12 and J558L transfectants (Figs 4 and 5*b*) shows a difference in the α/β complexes that are resolved in the nonreducing dimension. This may indicate that the α and β chains can form both (α/β) and (α/β)₂ disulphide-linked complexes; indeed, studies²⁶ on the polymerization of secretory IgM indicate that J558L and M12 differ in their efficacy at catalysing the formation of specific disulphides.

IgD surface expression

Cell lines that express IgD on the cell surface but which retain and degrade membrane IgM intracellularly^{24,27} may indicate that the IgD-associated proteins could not potentiate surface expression of IgM (ref. 24). That *mb-1* encodes the α chain of the IgD complex therefore came as a surprise. But our finding that membrane IgG2b can be transported to the surface of J558L in the absence of expressed *mb-1* suggested an alternative explanation. Perhaps IgD, like IgG2b, although able to associate with



α/β can nevertheless be transported to the surface in its absence. To test this, we transfect a δ_m plasmid lacking *mb-1* into J558L. Although we obtained an occasional clone which made membrane IgD but did not transport it to the surface, the large proportion of the transfectants stained for IgD on the surface (Fig. 6*a*) and this surface IgD had no associated heterodimer (Fig. 6*b*). Thus, both IgD and IgG2b can be transported to the surface of J558L cells without requiring *mb-1* coexpression. We introduced a δ_m , λ plasmid into nonlymphoid cells (NIH3T3 and COS-7) and a large proportion of the transfected cells expressed IgD on their surface (Fig. 6*c*), demonstrating that neither the α/β heterodimer nor other lymphoid-specific protein is needed for its cell-surface transport.

Discussion

Provision of the immunoglobulin-associated α and β chains in nonlymphoid cells allows transfected membrane IgM to be transported to the cell surface where it exists as an IgM/ $\alpha\beta$ complex; this supports a model in which the α/β heterodimer constitutes the sole core protein of the proreceptor. There may be other unidentified subunits in the complex and, indeed, more than two immunoglobulin-associated bands have been detected⁹⁻¹¹. But such additional subunits cannot be necessary for surface transport.

In contrast to previous expectations, these experiments indicate that the *mb-1* gene product associates with all five immunoglobulin classes and the classes therefore share a

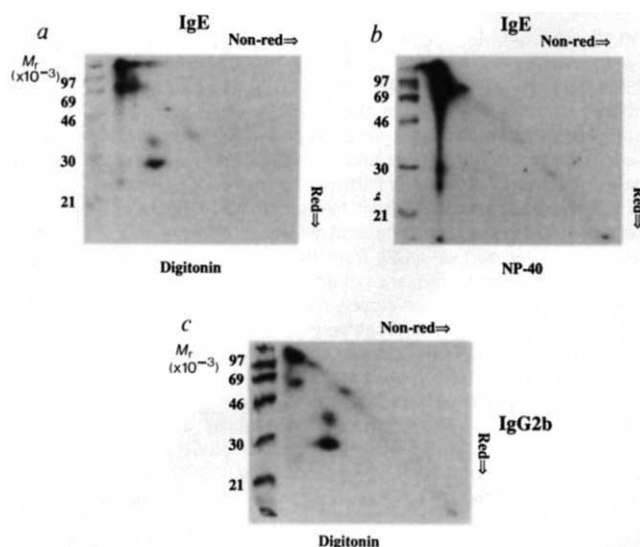
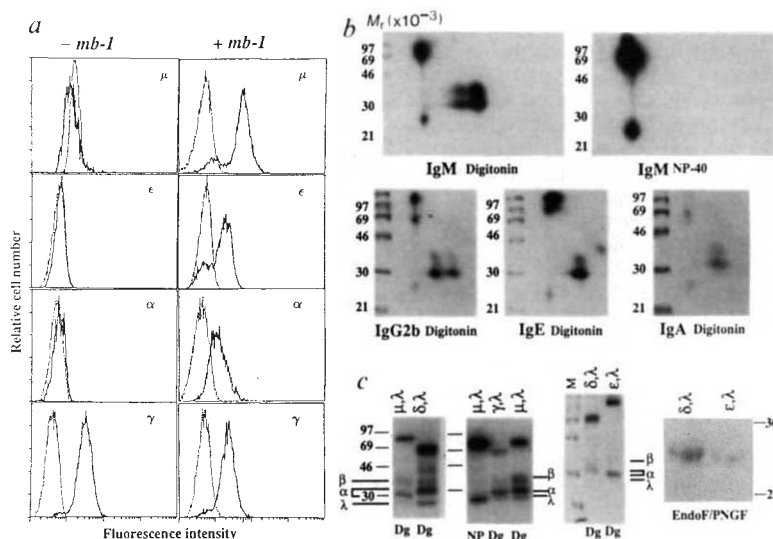


FIG. 4 Proteins associated with surface IgE and IgG2b in transfectants of the M12 B lymphoma. Anti-NIP antibody was purified on hapten columns from surface radiiodinated M12[ϵ_m , λ] (*a*) or M12[$\gamma 2b_m$, λ] cells (*b*) that had been lysed in buffer containing digitonin or NP-40 as indicated and subjected to two-dimensional SDS-PAGE.

FIG. 5 Association of the *mb-1* gene product with membrane IgM, IgG2b, IgE and IgA and its potentiation of surface immunoglobulin transport in plasmacytoma. **a**, Plasmacytoma J558L cells were transfected with plasmids that either just directed the expression of μ_m , γ_2b_m , ϵ_m or α_m IgH chains or with plasmids that also included the *mb-1* transcription unit; surface-stained cells (solid lines) were analysed on the FACS along with control cells (dotted lines). **b**, Analysis by two-dimensional SDS-PAGE of proteins associated with surface immunoglobulin in surface-radiolabelled J558L transfectants. The IgM complex is sensitive to NP-40 as shown in the top right panel. **c**, Comparison by one-dimensional SDS-PAGE of surface-labelled immunoglobulin-associated proteins in the J558L transfectants of different isotypes. The bottom right hand panel shows the mobility of the α chains of the IgD and IgE complexes after deglycosylation.

METHODS. Staining in the FACS for IgM, IgA and IgG2b was with FITC-conjugated class specific antisera (Southern Biotechnology), whereas the IgE λ protein was detected with the Ac38 IgG κ anti-idiotypic antibody³² and FITC-conjugated anti-mouse κ . Cytoplasmic immunofluorescence analysis revealed that, in each population of transfectants, at least 80% of the cells stained intracellularly for the transfected IgH chain.



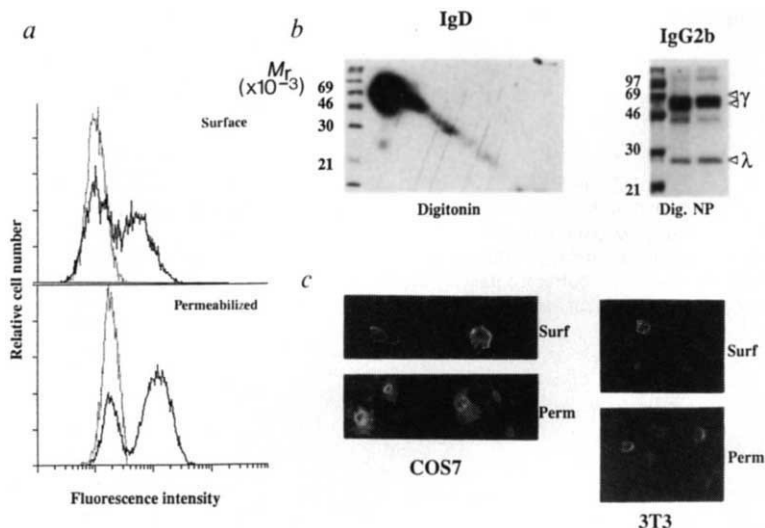
common associated α subunit. Our results are consistent with previous proposals²⁴ that the β chain does not exhibit an isotype-specific structure. We thus propose that all five immunoglobulin classes share a common proreceptor composed of the α/β heterodimer. The precise stoichiometry of the complex is unknown although the two distinct bands of α/β suggest that the complex may have an IgH₂L₂(α/β)₂ structure. The pattern of glycosylation of the α subunit seems to depend on the isotype with which it is associated. Thus, the α subunits associated with IgM, IgE and IgG have similar mobilities before deglycosylation whereas the α chain of IgD migrates distinctly slower in SDS-PAGE. Interestingly, Chen *et al.* have previously shown that the pattern of IgD-associated bands differs among the lymphoid organs and that this heterogeneity is at least partly attributable to glycosylation differences¹⁰. Thus, the pattern of Ig- α glycosylation may vary not just according to the class of the associated immunoglobulin molecule but also from tissue to tissue. The mechanisms responsible for the differences in glycosylation are unknown.

There are clear differences between the immunoglobulin classes in their dependence on the α/β heterodimer for surface transport. IgM cannot be transported to the surface in its absence and this requirement is stringent^{7,27}. By contrast, IgG can be detected on the surface of several plasmacytomas^{27,28}, cells which do not express *mb-1*, and we have described plasma-

cytoma transfectants which transport IgD and IgG2b to the surface without associated heterodimer. Curiously, there is clonal variation among the transfectants as some of the J558L cells express the transfected IgD but degrade it intracellularly; it was, presumably, this latter type of J558L[δ_m] transfectant which was studied by Wienands *et al.*²⁴. We have no simple explanation for this clonal heterogeneity. It cannot, however, be ascribed to a high frequency reactivation of *mb-1* expression as the sIgG2b⁺ and sIgD⁺ cells do not have any heterodimer associated with the surface immunoglobulin. Moreover, non-lymphoid δ_m λ transfectants express the IgD on the cell surface. Thus, whereas IgM seems to require the heterodimer to sheath hydrophilic residues in its transmembrane segment¹⁸, IgD must either not require such a sheath or other, non-lymphoid-specific components can substitute functionally for it. We have no data which allow us to discriminate between these alternatives. An interesting analogy²⁹ may be the surface expression of TCR β chain in cells lacking CD3.

All five immunoglobulin classes seem to share a common α/β proreceptor. It will be important to determine whether the differential glycosylation of the associated α chains has importance in immunoglobulin signalling or whether there remain other unidentified class-specific membrane immunoglobulin-associated molecules. That IgD can, unlike IgM, be transported

FIG. 6 IgD does not require lymphoid-specific proteins to be expressed on the cell surface. **a**, FACS profiles of J558L[pSVgpt- δ_m] and J558L control cells (dotted line) stained with anti- δ antiserum; the top panel shows surface stained cells whereas the bottom panel shows cells that have been fixed with formaldehyde and permeabilized with NP-40 before staining¹⁸. **b**, Analysis by two-dimensional SDS-PAGE of radiolabelled surface IgD from the transfected J558L[δ_m] cells and a comparison on a one-dimensional gel of radiolabelled and reduced surface IgG2b from J558L[γ_2b_m] cells that have been lysed in the presence of either digitonin or NP-40. γ_2b chains give rise to two closely migrating H-chain bands owing to glycosylation heterogeneity²⁶. **c**, Immunofluorescence of NIH3T3 and COS-7 cells transfected with a pSV-gpt- δ_m λ plasmid and stained on the surface with anti- δ antiserum. The α/β -independent surface transport of IgD was confirmed in multiple transfectants of several cell lines and the sequence of the δ transmembrane in pSV- δ_m was confirmed as unmutated by DNA sequencing. COS-7 cells were transfected by use of DEAE-dextran.



to the cell surface without associated α/β could be important in B cell development. The coexpression of IgD with IgM could compete for α/β and decrease the efficiency of IgM surface transport; the existence of a form of surface IgD that lacks

associated α/β could have implications for transmembrane signalling. Clearly, it will be necessary to ascertain whether surface immunoglobulin lacking associated heterodimer exists *in vivo*. □

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LETTERS TO NATURE

Correlation of magnetic and optical structure in the barred spiral galaxy M83

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THE barred spiral (SBc) galaxy M83 was previously studied thoroughly in the optical regime^{1–3}, and is known to possess an ordered magnetic field, with radio emission showing linear polarization of up to 60% in the outer regions^{4–6}. Earlier radio studies were done at lower frequencies, where Faraday rotation of the polarization by the magnetic field is important, but we have now observed M83 using the 100-m Effelsberg telescope at a frequency of 10.6 GHz, at which Faraday rotation should be less than 1°. There is significant polarization in the inner part of the galaxy, where we find that the magnetic field closely follows the optical structure. We see some deviations from the ordered magnetic structure that coincide with optical patchiness, and two pronounced polarization minima that correspond with enhancements of CO emission⁷. Our results suggest that the inner structure of the galaxy is more orderly than earlier work⁶ had indicated, and that any gross disorder, perhaps due to star formation, occurs only in the innermost regions. In addition, the observed distribution of Faraday rotation across the galaxy, deduced from comparison of observations at different frequencies, provides evidence that a significant component of the magnetic field extends away from the disk.

The observations were made in spring 1990 using a new four-feed receiver system operating at 10.6 GHz and installed in the secondary focus of the 100-m telescope. The observational technique and data analysis used the multi-beam restoration technique⁸ and the software beam-switching mode⁹. Each feed is followed by a two-channel receiver and a polarimeter, thus providing full Stokes parameters. A total of 11 coverages of an area that sufficiently covered the galaxy provided maps with sensitivities of 1.3 mJy per beam area and 0.6 mJy per beam area in total power and linear polarization, respectively. A more detailed description of the system and the data handling is given elsewhere¹⁰.

Figure 1a shows the $\lambda = 2.8$ cm total intensity map of M83 and Fig. 1b shows the polarized intensity and the 'E'-vectors. The total intensity emission is dominated by the central source which is superimposed on a bright radio disk. The detailed morphology of the total intensity map at $\lambda = 2.8$ cm agrees with the observation⁶ at $\lambda = 20$ cm with the D-array of the VLA.

In linear polarization a basic difference between the $\lambda = 2.8$ cm and the $\lambda = 6.3$ cm polarization data is evident, especially when inspecting Fig. 1b, which shows the $\lambda = 2.8$ cm map of the linearly polarized intensity. In contrast to the $\lambda = 20$ cm (ref. 6) and the $\lambda = 6.3$ cm map (ref. 5), Fig. 1b shows a considerable increase of linearly polarized emission towards the central region of M83. In view of the instrumental polarization of $\leq 1\%$ this polarized emission in the centre of M83, which is $\sim 10\%$, must be regarded as real. The highest percentage of polarization occurs in the outer spiral arms of M83, in agreement with previous lower-resolution studies^{5,6}, with values of up to 60%.

Figure 2 shows the projected magnetic field orientations overlaid on an optical image. To this end the 'E'-vectors have been rotated by 90°, as we anticipate little Faraday rotation at this wavelength. A preliminary evaluation of the rotation measure (RM) derived between $\lambda = 6.3$ and $\lambda = 2.8$ cm reveals that the vectors represent the intrinsic magnetic field orientation to within 1° ($|RM|_{\max} \leq 100$ rad m⁻², see below).

It is obvious from Fig. 2 that the projected magnetic field follows the spiral structure of M83 very closely and can be traced from the outermost diffuse spiral arms into the very central region. Even the abrupt changes in the pitch angle occurring at the transitions between the bar and the spiral arms are well reproduced by the magnetic field.

We have calculated the average degree of polarization of M83 at $\lambda = 6.3$ and $\lambda = 2.8$ cm. The thermal contribution to the radio emission was derived from the total spectrum and accounted for in this computation. The derived values are 12 and 13% at $\lambda = 6.3$ and $\lambda = 2.8$ cm, respectively (after smoothing the $\lambda = 2.8$ cm map to the same beam as that at $\lambda = 6.3$ cm)⁵. The near identity of the two values suggests that the depolarization producing the depression of the polarized emission in the central region of M83 is beam depolarization, being wavelength independent. The high-resolution maps of Ondrechen⁴ at $\lambda = 20$ and $\lambda = 6$ cm, which disclose linear polarization in the centre and along the bar of M83, corroborate this view.

Two 'holes' in polarization at $\lambda = 2.8$ cm (Fig. 1b) are not centred on the bar itself but are coincident with dust lanes which

emerge perpendicular to the bar. They are most probably caused by beam depolarization, assuming that the small-scale magnetic field structure also follows these dust lanes. The CO(1-0) distribution⁷ is characterized by a peak in the nucleus and secondary maxima $\pm 1'$ away, along the bar. These concentrations of CO coincide with the two small polarization 'holes'. This would suggest that in CO concentrations the magnetic field becomes tangled, possibly by turbulent motions of the molecular clouds, leading to strong beam depolarization.

At $\lambda = 20$ cm and $\lambda = 6.3$ cm, however, Faraday depolarization is also present, probably as a result of increased thermal electron density and magnetic field strength. The smoothed $\lambda = 2.8$ cm map gives a degree of polarization of $\sim 4\%$ in the central region, whereas at $\lambda = 6.3$ cm this value is only $\sim 1.5\%$. Wavelength-dependent depolarization of $p_{6.3}/p_{2.8} \approx 0.4$ could be the result of Faraday dispersion in a turbulent medium with cells of ~ 50 pc size containing thermal gas in the central region of $\sim 0.1 \text{ cm}^{-3}$ electron density and random magnetic fields of $\sim 20 \mu\text{G}$ strength. At $\lambda = 2.8$ cm Faraday dispersion is negligible, whereas at $\lambda = 20$ cm it is the dominating depolarization effect.

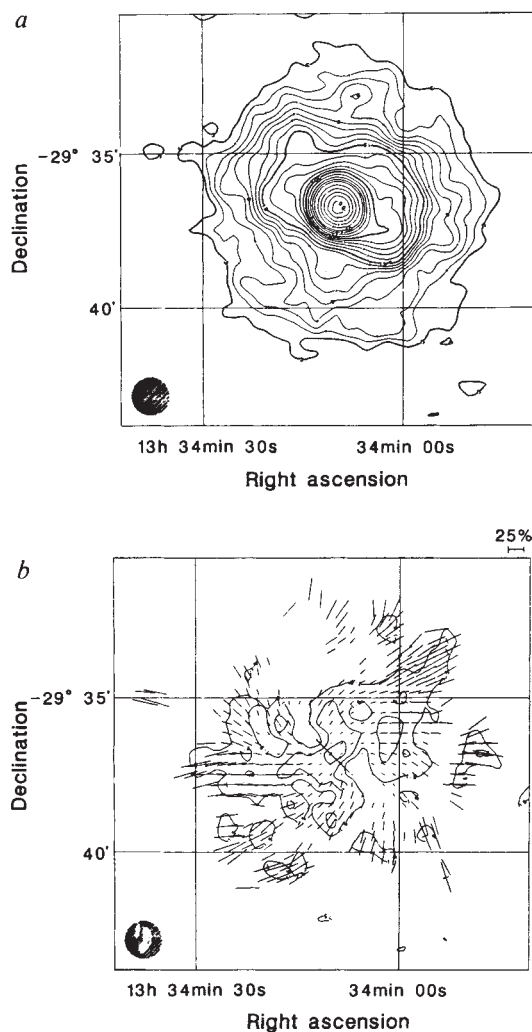


FIG. 1 *a*, Map of the total intensity of M83 at 10.6 GHz (2.8 cm). Contours are: 3.0, 4.5, ..., 15, 18, ..., 30, 35, ..., 50, 60, ..., 100 mJy per beam area. The hatched circle in the lower left indicates the beam size. *b*, Map of the linearly polarized intensity of M83 at 10.6 GHz. Contours are: 1.5, 2.5, 3.5 mJy per beam area. Superimposed are the 'E'-vectors, with their lengths proportional to the degree of polarization. The orientation of the bar is indicated. The beam size is indicated by the hatched circle in the lower left, the bar in the upper right calibrates the fractional polarization.

The overall picture arising from these polarization characteristics is that the magnetic field is highly disordered in the inner region of M83. This may arise because the star-forming activity is higher in the central region of this galaxy as evidenced in the H α maps, giving rise to increased turbulence and heating in the interstellar medium (ISM)⁵. It is a conspicuous feature that the observed soft X-ray emission¹¹ occupies essentially the entire region of M83 that is depolarized with larger beams. Farther out the magnetic field must be well aligned, as suggested by the high degrees of polarization ($\approx 60\%$).

The flux densities of the total and polarized synchrotron emission can be used to obtain an estimate of the average strengths of the random and uniform magnetic field components assuming equipartition between the energy densities of cosmic rays and the magnetic field, and a disk thickness of $1,500 \pm 500$ pc. Integrating our maps out to $7'$ we derive $S_{\text{tot}} = 632 \pm 40$ mJy and $S_{\text{pol}} = 61 \pm 8$ mJy at $\lambda = 2.8$ cm. From this we obtain $10 \pm 2 \mu\text{G}$ for the random and $4 \pm 1 \mu\text{G}$ for the uniform field components, yielding an average strength of the total field of $11 \pm 2 \mu\text{G}$.

We have performed a preliminary analysis of the Faraday rotation between $\lambda = 6.3$ and $\lambda = 2.8$ cm. A systematic variation of the rotation measure RM is seen across M83 with RM changing its sign in different quadrants of the galaxy, and absolute values of RM exceeding 100 rad m^{-2} . We take this as strong evidence for a magnetic field component perpendicular to the disk of M83, given its low inclination. At 90° inclination a uniform field of $\approx 4 \mu\text{G}$ strength (see above) and a thickness of $\sim 1\text{--}2$ kpc of the ionized disk with a 0.05 cm^{-3} thermal electron density can explain the observed rotation measures without enhancing the polarized intensity. If so, the observed distribution of RM would delineate a large-scale variation of the vertical component of the magnetic field. The holes in the distribution of the polarized brightness are accompanied by steep gradients in the rotation measures. It means that in the vicinity of the bar's terminations the vertical direction of the magnetic field changes abruptly.

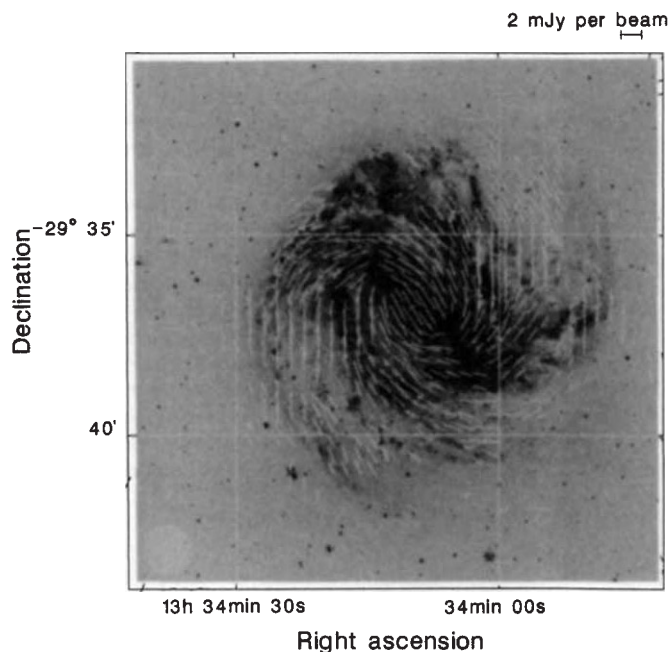


FIG. 2 Magnetic field orientation of M83, with 'B'-vectors at 10.6 GHz superimposed on an optical photograph by D. Malin (courtesy Anglo-Australian Observatory). The vectors delineate the orientation of the magnetic field. Their lengths are proportional to the polarized intensity, which is calibrated by the bar in the upper right. The hatched circle in the lower left corner indicates the beam size.

The possibility that the distribution of the rotation measure in M83 traces (at least partly) the magnetic field in the vertical direction would have important consequences for the study of the magnetic field morphology in galaxies. Any analysis of the RM distribution aiming at the determination of the large-scale disk magnetic field (see examples in ref. 12) will have to consider this effect, in particular in the case of mildly inclined galaxies. Vertical field components have been detected, for example in NGC6946¹³. On the other hand, it would provide a powerful tool to complement polarization observations of galaxies seen edge-on^{14,15}, which aim at the direct detection of the magnetic field in the haloes of galaxies. This would have a considerable impact on models describing galactic dynamics and would add to our growing knowledge about the relation between disks and haloes of galaxies. □

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The origin of the planet orbiting PSR1829–10

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BAILES *et al.*¹ have reported the discovery of a planet-mass object in a six-month orbit around the radio pulsar PSR1829–10. The parameters of the orbit, in particular its circularity, make it unlikely that this planet existed before the supernova explosion that presumably created the pulsar, and somehow survived¹. Here we present two alternative explanations for the existence and orbital parameters of the claimed planet-mass object. In the first, the pulsar forms from the coalescence of two white dwarfs, and the planet condenses from a massive disk of material left behind. In the second, a neutron star collides with and cannibalizes the central star of a solar-type planetary system. The orbits of the inner planets are made elliptical by this collision, but then recircularized by drag forces due to the extended but short-lived envelope of the disrupted central star.

An ordinary type II supernova explosion ejects more than half its original mass, and so no pre-existing planets would survive in bound orbits. Nor could planets form after such an event: a small fraction of the inner envelope ($M \leq 10^{-3} M_{\odot}$; ref. 2) could fall back towards the newborn neutron star, and might form a short-lived disk, but such a disk would not have sufficient angular momentum to form a planet-mass object at a distance of ~ 0.7 AU (which is the distance implied by the orbital parameters of the putative planet). There is, however, another way a neutron star might form which could create a massive disk ($M_{\text{disk}} \geq 0.1 M_{\odot}$) around it. This scenario involves two white dwarfs in a close binary system. The binary shrinks owing to

gravitational radiation; the lighter white dwarf is disrupted when it overflows its Roche lobe, forming, in the process, a disk around the more massive component³. The more massive white dwarf then accretes at a rate close to the Eddington rate ($\dot{M} \sim 10^{-5} M_{\odot} \text{ yr}^{-1}$), experiencing a series of off-centre carbon flashes which transform its composition from C–O to O–Ne–Mg⁴ (this phase is missing, if the primary is already an O–Ne–Mg white dwarf). When the O–Ne–Mg white dwarf reaches the Chandrasekhar mass (after $\sim 10^4$ – 10^5 yr), it collapses and forms a neutron star. This neutron star could still be surrounded by a disk, which takes up the excess angular momentum. Initially, the disk is massive ($M_{\text{disk}} \sim 0.1 M_{\odot}$) and with radius $R_{\text{disk}} \sim 10^9$ cm corresponding to the orbital angular momentum of the lighter white dwarf at the time of its disruption. It is likely to be self-gravitating and to evolve rapidly. By the time it has evolved to a disk radius of 1 AU, its mass will be $10^{-2} M_{\text{disk}} \sim 10^{-3} M_{\odot}$. At that stage the disk closely resembles models for the presolar nebula except that it has somewhat less total mass, but rather more mass in heavy elements. The temperature structure may also be similar. Planets could form in such a disk by the same processes by which they formed in our own Solar System, except that they would all be ‘terrestrial’ and contain no light elements.

One of the chief uncertainties of this scenario is whether the coalescence of two C–O white dwarfs leads to core collapse or a thermonuclear explosion. In the latter case, the star would probably be completely disrupted without leaving a compact remnant—experience a type Ia supernova⁵. Which of the two possible outcomes takes place depends mainly on the accretion rate in the disk-accretion phase. Calculations⁶ show that core collapse, leading to the formation of a neutron star, occurs when the accretion rate is larger than $\sim 1/5$ the Eddington rate.

This first scenario explains not only the existence of a planet around a pulsar, but also the system’s location very close to the galactic plane, as the progenitor stars would be relatively massive ($M \geq 4 M_{\odot}$)—that is, they belong to a typical young disk population. Very little mass is ejected during accretion-induced collapse, so there is less likelihood that a pulsar formed by this route would acquire a large velocity. (Moreover, even if non-spherical collapse did impart an impulsive recoil velocity of $\sim 100 \text{ km s}^{-1}$, the newly formed pulsar would not break loose from the disk, as the latter is then very tightly bound, with radius $\sim 10^9$ cm and orbital velocity $\sim 3,000 \text{ km s}^{-1}$).

In our second scenario, we propose that the planet did not form around the neutron star or its progenitor, but instead in a solar-type system resembling our own; this system later experienced a catastrophe when a neutron star passed close enough to its ‘sun’ to merge with it. This process requires that the neutron star approaches within a ‘perihelion’ distance of ≤ 2 ‘solar’ radii. It is therefore no more improbable than the widely discussed process of tidal capture for the formation of low-mass X-ray binaries (LMXBs) and millisecond pulsars⁷, which requires a neutron star to pass as close as 2–3 stellar radii to a main-sequence star. Such collisions are most frequent in globular clusters⁸. At present, it does not seem to be ruled out that PSR1829–10 is actually in a globular cluster, as its position in the sky is in a highly obscured region of the galactic disk where a globular cluster could easily have escaped detection (see, for example, ref. 9). Could ‘solar systems’ survive in a globular cluster? A planet in an orbit of ~ 1 AU would be dislodged from its orbit if another star passed within ~ 2 AU—for more distant encounters the orbit would respond adiabatically. The relevant timescales are in proportion to the standard ‘Spitzer’¹⁰ time, t_{R} , for stellar relaxation. For a stellar velocity dispersion of 10 km s^{-1} , a star would typically have to wait $\sim 50 t_{\text{R}}$ before another came within 2 AU. The timescale for the evolution of the cluster’s radial structure is $\sim 100 t_{\text{R}}$, so most ‘inner planets’ could survive for a time of the order of the dynamical-evolution timescale of the cluster. By scaling the collision rates to the observed numbers of LMXBs in globular clusters, we estimate

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that several direct collisions of neutron stars with solar-type systems could occur during the lifetime of a compact cluster. (A neutron star could capture a planet by a three-body exchange reaction even if it got no closer than ~ 2 AU. Such an event may be 100 times more probable than the close encounters considered here, but it is much less likely to yield the kind of system Bailes *et al.* have discovered: there is only a 1% chance that the captured planet would orbit the neutron star with an eccentricity of < 0.1 ; nor is there any reason why the neutron star should then be reactivated as a pulsar.)

In the galactic disk, collisions or close encounters will be much rarer than in globular clusters. The total collision rate for the Galaxy is mainly determined by the total number of slow neutron stars (neutron stars with velocities $v_n \sim 20 \text{ km s}^{-1}$). We estimate that, for a population of 10^8 slow neutron stars in the Galaxy, there will be one such collision every $3 \times 10^8 \text{ yr}$ (where we assumed that planetary systems are common). In addition, a population of 5×10^8 fast neutron stars (with $v \sim 100 \text{ km s}^{-1}$) will contribute a similar event rate.

The collision of a neutron star with the central sun of a solar-type system has two main consequences: one, the sun is completely disrupted¹¹, with its constituent material becoming a very distended envelope around the neutron star; and two, the outer planets are lost from the system. The inner planets (for which $v_p/v_n \geq 0.3$, where v_n is the neutron star velocity at infinity and v_p the planet's orbital velocity) can remain bound, although their orbits are likely to acquire a substantial eccentricity.

If a neutron star passed inside a main-sequence star (or indeed was tidally captured by a first passage that brought it to within ~ 2 stellar radii) the system's angular momentum would be too small to allow it to evolve into a synchronously rotating binary (or a LMXB). Instead, the neutron star would quickly be dragged to rest in the whole system's centre-of-mass frame, the combined angular momentum of the two stars being stored in an envelope composed of the material of the disrupted main-sequence star, most of which remains bound¹¹. The system then constitutes a Thorne-Zytkow object (TZO)¹²—namely, a supergiant-like star (with a radius $R \approx 4 \text{ AU}$; R. Cannon, personal communication) with a neutron core, radiating at the neutron star's Eddington luminosity ($L \approx 5 \times 10^4 L_\odot$). Because its radius is so much larger than the original star's, the envelope of the TZO can still be roughly spherical despite the angular momentum it contains. The envelope is extensive enough to engulf the innermost planets. Owing to drag forces, the orbits of the engulfed planets will start to decay and to be recircularized.

The lifetime of the TZO phase is mainly determined by how rapidly the star loses its envelope in a stellar wind. Adopting a Reimers mass-loss rate, and assuming an envelope mass of $1 M_\odot$, we estimate that its lifetime is $\sim 10^5 \text{ yr}$. This is comparable with the characteristic drag timescale on which the orbit of an engulfed Earth-like planet would decay. The orbits of inner planets should therefore shrink substantially. The timescale depends of course on the orbital parameters, and, for a given orbit, it scales roughly as the cube root of the planet's mass.

The circularization timescale depends on whether the planets are on photosphere-crossing orbits or completely within the TZO envelope. If the orbits are well below the photosphere, circularization is relatively inefficient (because low-mass TZO envelopes have very small density gradients¹²). Typical circularization times are then comparable with the drag timescale.

Planets for which the latter is similar to the lifetime of the TZO phase ($\sim 10^5 \text{ yr}$) should thus retain a finite, albeit small eccentricity. On the other hand, if a planet were initially on a photosphere-crossing orbit (as Jupiter and perhaps Mars would be), then the large density gradient near the photosphere would ensure efficient circularization, even without much reduction in periastron distance. It is unlikely that there are other planets interior to the presently known planet (as the spiral-in time would be shorter than the lifetime of the TZO phase). Planets with longer periods are likely to be in eccentric orbits: in particular, the orbits of planets with periastron distances $\geq 4 \text{ AU}$ should still reflect the orbits they attained immediately after the initial collision. The orbits of different planets are not likely to be coplanar, as they are all affected differently by the previous collision (unless the neutron star's trajectory of approach lay in the ecliptic of the original solar-type system).

The ambient temperature within the TZO envelope would be of order 10^4 K . Low-mass planets composed of light elements would evaporate within $\sim 10^5 \text{ yr}$, the evaporation rate being controlled by the rate at which heat can be conducted into them. However, a planet of $> 10 M_E$, where M_E is the mass of the Earth, would survive: not only would the conduction be slower, but, especially if it were made of heavy elements, its self-gravity would be important even if its interior were heated to the ambient temperature. The central neutron core, accreting at its Eddington rate, would gain $\sim 2 \times 10^{-3} M_\odot$, if the TZO phase lasted for 10^5 yr . An important consequence of this is that a TZO phase can revive an old neutron star as a pulsar. Indeed, the expected accreted mass is more than enough to spin-up the neutron star to the observed period of $\sim 300 \text{ ms}$; the qualitative details depend on how angular momentum is redistributed within the TZO envelope as it evolves. (In this connection, we note that the envelope may not be quasi-spherical in its final evolutionary stages¹³. If some residual material deflated into an extended bound disc, 'secondary' planets could then condense, rather as in our first scenario, whether or not the original main sequence star had a solar system around it.)

It is difficult to validate either of the two models discussed above, but future observations will undoubtedly provide further insights into this extraordinary system. Important issues are: the possible membership in a globular cluster, the system's space velocity, the circularity of the planet's orbit, the presence and orbits of other planets and the magnetic field of the central neutron star. In a white-dwarf merger model, all the planets should be in circular, coplanar orbits, whereas in a capture scenario any pre-existing outer planets would be in eccentric, oblique orbits.

In conclusion, although it is difficult to form a neutron-star planet system as may be observed in PSR1829-10, it is by no means impossible. We have discussed two models that can lead to systems of this type, both of which should certainly occur in nature. In either case, the pulsar would be expected to be unusual—either it formed through an unusual route, or it has been resuscitated in an unusual way. In the first scenario, the 'planet' formed in an unusual fashion from a disk of material spun off a coalescing white-dwarf binary. If the second scenario, on the other hand, proves to be correct, then this would be exciting because it would imply that planetary systems must be common: only a small fraction of solar systems, fortunately, could experience the kind of catastrophe that gave rise to PSR1829-10. $\square$

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Unusual interaction of the high-velocity pulsar PSR1757–24 with the supernova remnant G5.4–1.2

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THE peculiar fan-shaped morphology of the galactic radio source G5.4–1.2 has prompted much speculation about its nature^{1–4}. Here we report high-resolution observations obtained with the Very Large Array, which reveal a compact, highly polarized, flat-spectrum radio nebula on the western edge of G5.4–1.2. We also confirm the presence of a 125-ms pulsar⁵, PSR1757–24, associated with the radio source, and find that it is located near the centre of the newly discovered nebula. We argue that the pulsar is associated with G5.4–1.2, which we identify as a supernova remnant, and that it was born with a high enough velocity, $\sim 2,000 \text{ km s}^{-1}$, for it to overtake the decelerating supernova shell, creating the peripheral radio nebula. The fortuitous near-coincidence of the pulsar velocity with the shell velocity accounts for the unusual overall shape of the remnant. If the large velocity of the pulsar is confirmed by future observation of its proper motion, the possibility of asymmetric supernova explosions must be taken seriously, and other previously unsuspected associations between pulsars and supernova remnants may emerge.

G5.4–1.2 is a fan-shaped radio nebula with edge-brightened emission along an incomplete arc towards the west. On the basis of the observed high linear polarization and the negative spectral index, the nebula has been classified as a supernova remnant¹ (SNR). A compact source, G5.27–0.90, lies just outside the western arc and is apparently connected to G5.4–1.2 by a bridge of emission. This bridge and G5.27–0.90 seem to define an axis of symmetry. These features led Helfand and Becker^{2,3} to suggest that the entire nebula was not a SNR but the prototype of a new class of nonthermal nebulae powered by relativistic particles generated by a rapidly moving, accreting neutron star binary system, for example Cir X-1⁶.

This interpretation was disputed⁴ when a wide-field image at 843 MHz revealed that the western arc was complemented by a faint eastern arc, suggesting that G5.4–1.2 was a shell SNR (Fig. 1a). Furthermore, the short-period pulsar PSR1758–24 (period $P = 125 \text{ ms}$) had a positional error circle of radius $13'$ (90% probability) which enclosed G5.27–0.90 and overlapped G5.4–1.2 (ref. 5). The pulsar is also located at a distance of 6 kpc, similar to that assumed for G5.4–1.2 (archival VLA H_i absorption data indicate that G5.27–0.90 must be at a distance of $\geq 5 \text{ kpc}$; we adopt a distance of 6 kpc throughout). Thus Caswell *et al.*⁴ speculated that PSR1758–24 was born at the centre of G5.4–1.2 and that it is currently powering G5.27–0.90. The authors realized that the pulsar would have to be exceedingly energetic and thus necessarily young ($\sim 10^4 \text{ yr}$). The displacement of the pulsar from the centre of G5.4–1.2 would then require an improbable transverse speed of $2 \times 10^3 \text{ km s}^{-1}$. In contrast, accreting neutron star binaries can produce Eddington-limited luminosities for several hundred million years—a point in favour of the Helfand–Becker hypothesis.

Given the importance of unravelling the origin of this enigmatic SNR, and also to elucidate the relation of PSR1758–24 and G5.4–1.2, we undertook several new observations. In August 1990 we used the Very Large Array (VLA) in its B-array

configuration to observe a field centred on the nominal position of the pulsar, at wavelengths of 20 cm and 6 cm. At 20 cm, we clearly see that G5.27–0.90 consists of an elongated trail of emission connecting the western arc of G5.4–1.2 to a compact object. Linear polarization is detected over most of the region in Fig. 1b. The measured fractional polarization for the compact object is 20% and is probably only a lower limit, as we see evidence of considerable Faraday rotation.

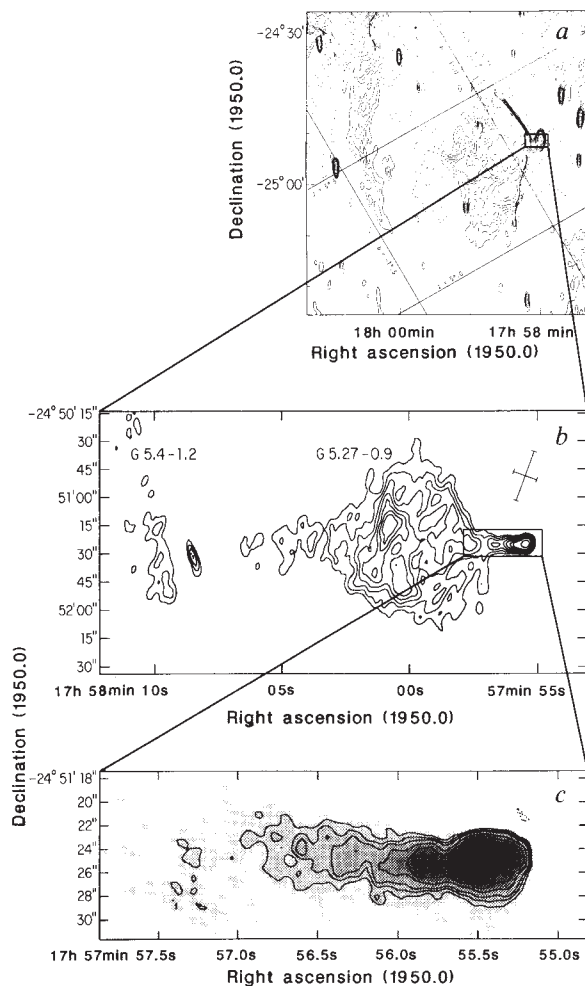


FIG. 1 a, A radio image of G5.4–1.2 and G5.27–0.90 at 843 MHz taken by Caswell *et al.*⁴ with the Molonglo Observatory Synthesis Telescope and used here with the permission of the Royal Astronomical Society. The synthesized beam is $110'' \times 42''$. A bright H_{ii} region in the upper left corner protrudes into the faint eastern arc of G5.4–1.2. The boxed area encloses the region shown in b. b, A 20-cm image of G5.27–0.90 taken with the VLA B-array. The source is resolved into a compact source to the west and is connected to the western arc of G5.4–1.2 (labelled) by a bridge of emission. The image is the result of 160 min of observations with a 25-MHz bandwidth. Contour levels are 3, 5, 7, 9, 11, 13, 15 and 20 times the r.m.s. noise of $150 \mu\text{Jy}$ per beam. The peak intensity is 3.7 mJy per beam and the beam size (full width at half maximum, FWHM) is $7'' \times 3.8''$. The cross is the FWHM of the phased-array beam (see text) with which the search for a pulsed signal from the pulsar was made. The search location, and hence the nominal position of the pulsar, was at the radio maximum of the cometary nebula in this image. The boxed area encloses the region shown in c. c, A 6-cm image of the newly discovered comet-shaped object in G5.27–0.90. The image was made with data obtained by combining archival VLA data in A/B-array from 1985 with B-array data from 1990, for a total of 85 min of integration time. The contour levels are $-3, 3, 4, 5, 7, 9, 11, 13, 15$ and 17 times the r.m.s. noise of $45 \mu\text{Jy}$ per beam. The peak intensity is 0.9 mJy per beam and the beam size is $2'' \times 1.3''$.

The increased resolution of the 6-cm image (Fig. 1c) reveals that the compact source is a cometary-shaped structure with a radius of $\sim 2''$. The integrated flux of this structure is 9 ± 2 mJy at 6 cm and 10 ± 1 mJy at 20 cm. Within the errors, this corresponds to a flat spectrum source ($\alpha \approx 0$, where flux at frequency ν is $\propto \nu^{-\alpha}$). The morphology of the image presented in Fig. 1c (including the flat spectral index and high linear polarization) is strikingly similar to that of the compact nebula around the pulsar PSR1951+32 (period 40 ms) in the bizarre SNR CTB80^{7,8}. This reasoning led us to search for pulsations from the cometary-shaped structure using the VLA.

The analog sum of the phased VLA was sampled with a filter bank (14 channels of width 4 MHz in each sense of polarization) at the rate of 403.2 Hz per channel and recorded on magnetic tapes for a total period of 40 min (30 January 1991). The data analysis was carried out on a Convex C 1 computer at the Californian Institute of Technology. Dispersion was removed from the data at several dispersion measures, each of which was searched for pulsed signal. A pulsar with a period and a dispersion measure similar to PSR1758-24 was easily detected in a 2^{19} -point transform (see Fig. 2). Thus we conclude that PSR1758-24 (now appropriately renamed as PSR1757-24) is indeed powering G5.27-0.90.

The salient parameters of the pulsar and its profile are given in Table 1 and Fig. 2. The rate of change of period, $\dot{P}$, is estimated by comparing the period in 1991 with the discovery period in ref. 6. A more accurate value is given in Table 1, determined from an ongoing timing program (S. Thorsett, personal communication). Using this value of $\dot{P}$ we infer a characteristic age $t_c \equiv P/2\dot{P}$ of 1.6×10^4 yr and a rotational energy loss rate $\dot{E}_R$ ($=4 \times 10^{46} \dot{P} P^{-3}$) of 2.6×10^{36} erg s⁻¹. A great amount of timing noise seems to be present, as expected for such young pulsars.

A chance coincidence between G5.4-1.2 and PSR1757-24 does not seem likely. The common distance of 6 kpc for the pulsar and G5.4-1.2, and the morphological evidence linking G5.4-1.2 with G5.27-0.90 in which PSR1757-24 resides, provide strong support for a real physical association between all three objects. The entire nebula is thus reminiscent of PSR1951+32 and SNR CTB80. For this system it has been argued^{9,10} that the pulsar was born with a large velocity 'kick'. The pulsar eventually caught up with the swept-up shell and in the process 'rejuvenated' a portion of the SNR shell as well as powering a miniature synchrotron nebula similar to G5.27-0.90.

In the absence of a direct measurement of the velocity for PSR1757-24, we can consider two situations. In the simplest situation, we assume that the pulsar originated at the geometric

TABLE 1 Observable parameters for PSR1757-24

Right ascension (1950)*	17 h 57 m 55.483(7) s
Declination (1950)*	-24° 51' 24.8(1)''
Pulse period (msec)	124.8737
Period derivative (10^{-15} ss ⁻¹)†	148.7(1)
Epoch (MJD)‡	48287.10
Dispersion measure (pc cm ⁻³)	292.9 $\pm$ 4.3
Flux density at 1.67 GHz (mJy)	1

* Position of radio maxima for the cometary nebula at 20 cm.

† Secular $\dot{P}$. Early timing results give $\dot{P} = 127.78(6)$ (S. Thorsett, personal communication).

‡ Modified Julian day.

centre of G5.4-1.2, and has travelled 21' due west since its birth. If the magnetic field strength of the pulsar is constant in time, then t_c is the (maximum) age of the pulsar. Clearly, $t_s \approx t_c$, where t_s is the age of the SNR. The transverse velocity of the pulsar is $v_p \approx 2,300$ km s⁻¹, the highest velocity for any known pulsar. (Asymmetric expansion of the SNR would shift its geometric centre and could lower this velocity by as much as a factor of two.) The speed of the shell, v_s is thus $\sim 2 \times 10^3$ km s⁻¹ and the swept-up mass is $M_s \approx 8(n/n_0)M_\odot$ where n is the ambient particle density and $n_0 = 3 \times 10^{-3}$ cm⁻³. By balancing the momentum of the pulsar wind, $\dot{E}_R/4\pi r_w^2 c = nm_H v_p^2$ where $r_w \approx 0.06$ pc is the standoff distance for the cometary-shaped structure (Fig. 1c) and m_H is the mass of a hydrogen atom, we find $n \approx n_0$. M_s is comparable to the total mass lost (ejecta plus mass loss in the red and blue supergiant phase) by any reasonable progenitor of the pulsar. Thus, it is possible that the SNR shell, despite its large size, is not in a fully developed Sedov phase.

The low value we infer for n , the ambient density, is consistent with recent results on the absence of shells around young pulsars^{11,12} in the inner Galaxy. Such a low density is attributed¹² to the clearing action of previous supernovae and/or energetic stellar winds. Low ambient densities result in rapid expansion of the shell with attendant low internal pressure. The pulsar wind thus rapidly expands and undergoes large adiabatic losses, and the resulting plerion has an exceedingly low surface brightness. But in the case of G5.4-1.2, as in CTB80, the interaction of the pulsar with the faint SNR shell rejuvenates the radio emission.

The apparent coincidence of v_s and v_p accounts for the remarkable 'V' shape of the western shell. The coincidence ensures that over most of its current lifetime the pulsar is embedded within the shell, continuously pumping energetic particles and magnetic field into the shell. Thus spherical expansion is considerably modified and the shell becomes more the shape of a bow shock wave. The 10^{50} erg in particles and fields demanded by radio observations and minimum energy arguments² is readily accounted for by the spin-down energy of an initially rapidly rotating ($P \approx 10$ ms) pulsar. The transport of the particles to distant parts of the shell is still a problem, as in CTB80. If, however, there is bulk flow at velocities comparable to the post-shock dispersion, the problem is solved.

This model, although generally reasonable, has several difficulties. The very low mean interstellar density over such a large volume is difficult to accept. Although asymmetric explosions have been invoked in the past, the magnitude of v_p requires extraordinarily asymmetric explosions.

An alternative model is to let t_s be much greater than t_c , which is possible by having the pulsar magnetic field grow with time^{13,14}. In this case, $t_c \approx t_g/2$ where t_g is the timescale for exponential field growth. Assuming $t_s \approx 10 \times t_c$, we find that $v_p \approx 230$ km s⁻¹, about equal to the mean speed of radio pulsars¹⁵. Applying the momentum balance as before yields $n \approx 0.25$ cm⁻³. The SNR would clearly be in the snowplough phase. The estimated radius is $20(n/0.25)^{-0.26}$ pc, about equal to the

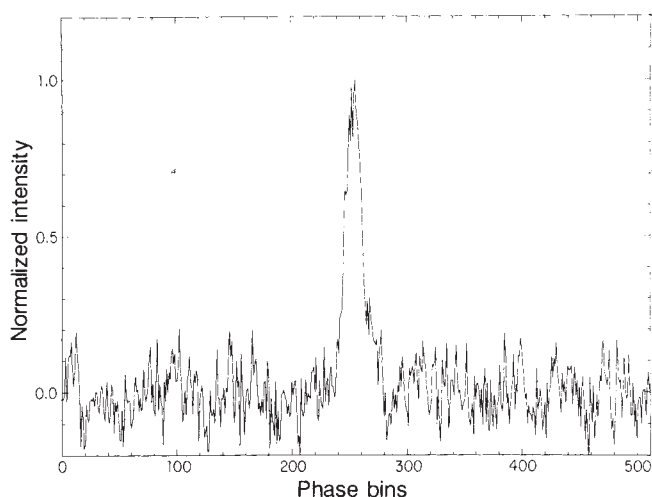


FIG. 2 Integrated pulse profile of PSR1757-24 obtained with the VLA at 1.67 GHz. The time resolution is 0.24 ms and the integration interval was 10 min.

observed radius. This model is suspect, however, because the trail of emission left by the pulsar and the high degree of asymmetry between the western and eastern arc suggest that the pulsar's true age is t_c and it is comparable to t_s .

Fortunately, a simple observational test will easily distinguish between the two models. In the first model (our preference) the proper motion of the pulsar will be $0.08'' \text{ yr}^{-1}$, which is easily detectable, whereas the other model has proper motion smaller by a factor of 10.

The interaction of high-velocity, active pulsars with their supernova shells or the ambient medium is expected to be a rather common phenomenon, and systematic searches at either X-ray or radio wavelengths should reveal new examples. G5.4–1.2 joins the emerging class of composite remnants⁹, which includes CTB80 and perhaps G359.1–0.5 (ref. 16), whose peculiar morphologies are produced by this form of interaction. G5.4–1.2 is probably an extreme example because of the close coincidence between its shell and the pulsar's velocity. $\square$

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Relation of structure and superconducting transition temperatures in A_3C_{60}

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THE discovery of conductivity¹ in A_xC_{60} (where A represents an alkali metal) and superconductivity² in K_xC_{60} has been followed by reports of superconductivity in other alkali-metal-doped fullerenes with transition temperatures as high as 33 K (ref. 3). Elucidation of phase diagrams and understanding the relationship between structure and superconducting properties is essential to a detailed understanding of superconductivity in these systems. So far, structural data have been reported only for the non-conducting, intercalated body-centred cubic (b.c.c.) structures A_6C_{60} (where A is K or Cs; ref. 4), a body-centred tetragonal structure for A_4C_{60} (where A is K, Rb, Cs; ref. 5) and the superconducting, intercalated face-centred cubic (f.c.c.) material K_3C_{60} (ref. 6). Here we report the preparation of a series of single-phase, isostructural f.c.c. superconductors with composition A_3C_{60} (where A is K, Rb, Cs or a mixture of these), and show that T_c increases monotonically with the size of the unit cell. Extended Hückel band-structure calculations also show a monotonic increase in the density of states at

the Fermi level, $N(E_F)$, with lattice parameter. The primary implication of these results is that all A_3C_{60} superconductors have the same structure and that changes in T_c can be accounted for by changes in $N(E_F)$.

A_xC_{60} samples were synthesized by reaction of C_{60} with stoichiometric quantities of alkali metals. The quantity of alkali metals was controlled volumetrically by cutting lengths of alkali-metal-filled capillary tubes in a dry box. The reagents were sealed in evacuated pyrex tubes and heated for two days at 225 °C, after which no alkali metal was visible in the tube. Annealing from 2 to 25 days while gradually increasing the temperature was necessary to attain equilibrium. Typical final annealing temperatures were 350 °C for samples containing potassium and 400 °C for other samples. Pelletizing the Rb_3C_{60} and Cs_3C_{60} enhanced the rate of the solid-state reaction, indicating that intergrain diffusion is important at 400 °C. The progress of the reactions was monitored by measuring the superconducting volume fraction (that is, the diamagnetic d.c. shielding) and the amount of f.c.c. phase present (by X-ray powder diffraction).

Representative Cu $K\alpha$ X-ray powder patterns for A_xC_{60} are shown in Fig. 1. The pattern for K_3C_{60} is in good agreement with that of Stephens *et al.*⁶ The other A_3C_{60} patterns are also readily indexed with isostructural f.c.c. cells. The relative intensities of the diffraction peaks are in agreement with those calculated on the basis of a spherical shell of charge for the C_{60} and occupation by the appropriate alkali-metal ions of the octahedral and tetrahedral sites. All A_3C_{60} samples shown in Fig. 1 are essentially single phase, with only minor inclusions of a second phase which we have identified as A_4C_{60} (ref. 5). We have not attempted to investigate possible ordering between octahedral and tetrahedral sites in the mixed alkali-metal systems, although one expects a preference of the larger A atom for the octahedral sites. In Table 1 we summarize the superconducting volume fraction, transition temperature T_c , and lattice parameter for the single-phase superconducting compositions studied, as well as a calculated density of states at the Fermi level, $N(E_F)$. The latter is discussed in more detail below. Of note is the absence both of superconductivity and of any diffraction evidence for an intercalated f.c.c. Cs_xC_{60} . We infer

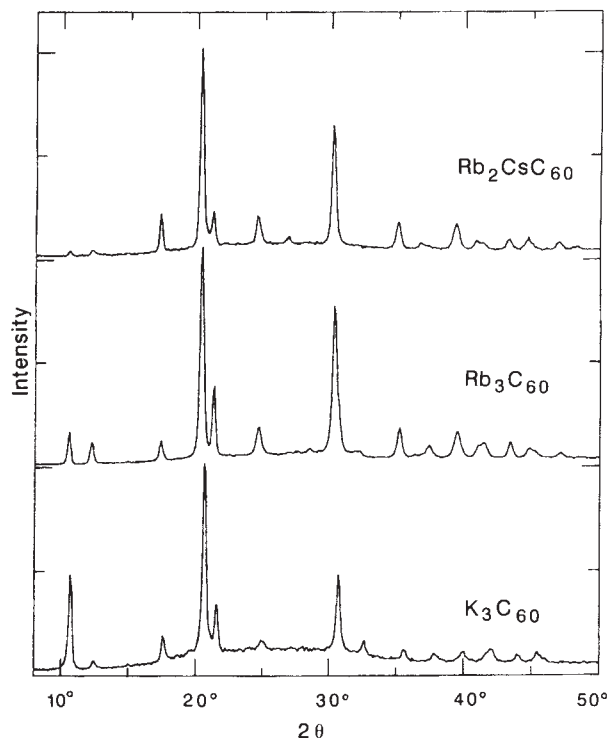


FIG. 1 X-ray powder patterns for K_3C_{60} , Rb_3C_{60} and Rb_2CsC_{60} .

from this that there is steric hindrance of incorporation of Cs, into the relatively small tetrahedral sites of the f.c.c. structure.

The superconducting transition temperatures of the A_3C_{60} compositions increase monotonically as the unit-cell size increases (Fig. 2). (Because of the small range of lattice parameters of the superconducting compositions, there is little change in the shape of the curve if the data are plotted as a function of volume rather than lattice parameter.) The range of T_c s observed for A_3C_{60} as a function of cell size is considerable, but not unusually large if we take $(\Delta T_c/T_c)/(\Delta V/V)$ as a relevant scaling factor. For A_3C_{60} this factor has a value of about 8. For comparison, both the organic β -(ET) $_2$ X compounds⁷ and the inorganic $Ba_{1-x}K_xBiO_3$ system⁸ have values near 22. The scaling factor is of the order of 1,000 for copper oxide superconductors⁹.

If the A_3C_{60} compounds have the same compressibility as C_{60} (refs 10, 11), these results predict a pressure dependence of T_c on the order of 2 K kbar⁻¹. The experimental values^{12,13} are about a third of this, perhaps reflecting a stiffening of the structure on alkali-metal intercalation. If we assume that correlation effects are negligible and that the T_c variation both in the pressure studies and in our volume studies arises from the same microscopic origin, then it is possible to estimate a compressibility for A_3C_{60} . Taking $\partial T_c/\partial P = -0.63$ to -0.78 K kbar⁻¹ (refs 12, 13) and $\partial T_c/\partial V = 0.088$ K Å⁻³, we obtain an isothermal compressibility of $(1/V) \times [\partial V/\partial P]_T \approx 2.5 \times 10^{-3}$ to 3.1×10^{-3} kbar⁻¹, compared with 5.5×10^{-1} kbar⁻¹ for pristine C_{60} (ref. 11).

We speculated previously¹⁴ that superconductivity in K_3C_{60} and Rb_3C_{60} resulted from electron pairing mediated by high-frequency intramolecular phonons of the C_{60} molecule, with the change in T_c between K_3C_{60} and Rb_3C_{60} explained by a change of the density of states at the Fermi level by about 10%. In this weak-coupling BCS model, the variation of T_c is given by

$$T_c = \omega_{ph} e^{-1/VN(E_F)}$$

where ω_{ph} is the energy of the relevant phonons (here 1,000–2,000 K)¹ and V is the electron-phonon coupling strength. We

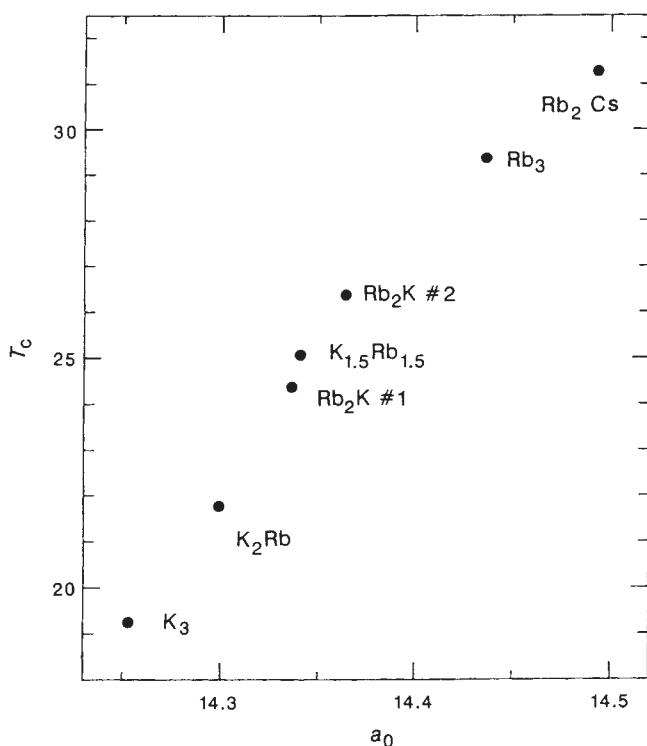


FIG. 2 Variation of the superconducting transition temperature T_c (K) with lattice parameter a_0 (Å) for various compositions of A_3C_{60} .

TABLE 1 Superconducting and structural parameters for A_3C_{60}

A_3^*	f.c.c. a_0 (Å)	$N(E_F)$ (states per eV per C_{60})	T_c (K)	Flux exclusion (d.c. %)
Rb ₂ Cs	14.493 (2)	23.9	31.30	48.0
Rb ₃	14.436 (2)	22.9	29.40	55.0
Rb ₂ K (#2)	14.364 (5)	21.7	26.40	32.0
Rb ₂ K (#1)	14.336 (1)	21.7	24.40	33.5
Rb _{1.5} K _{1.5}	14.341 (1)	21.3	22.15	47.0
K ₂ Rb	14.299 (2)	20.7	21.80	22.0
K ₃	14.253 (3)	20.1	19.28	28.6

* Nominal alkali metal composition. Estimated uncertainty $\pm 10\%$.

have estimated $N(E_F)$ using extended Hückel band-structure calculations. (A similar calculation for C_{60} was reported earlier¹⁵.) We used experimental lattice constants and calculated the density of states of each structure by using the two special points¹⁶ of the f.c.c. Bravais lattice. This approximation overestimates $N(E_F)$ by $\sim 50\%$, but should be adequate to allow comparisons among the isostructural A_3C_{60} compounds. The results are included in Table 1. The 10% variation of $N(E_F)$ required to produce the change in T_c is easily accounted for by the calculated values. This is in contrast to organic superconductors such as β -(ET) $_2$ X, for which similar calculations show that $N(E_F)$ is essentially constant¹⁷. Our results imply a negligible variation in the phonon frequency and electron-phonon coupling among the various A_3C_{60} compounds. This would be consistent with the idea of C_{60} intramolecular phonon modes being important in A_3C_{60} superconductivity, or with intermolecular modes that are weakly affected by the observed changes in lattice parameter. In either case, the density of states seems to be controlling changes in T_c .

Note added in proof. We have found that samples of nominal composition $Cs_{1+x}Rb_{2-x}C_{60}$ for $0 < x < 1.25$ have T_c s of 32.5 K, in agreement with ref. 3. In addition, X-ray diffraction shows these compositions to be multiphase with the f.c.c. fraction having a lattice parameter $a = 14.506$ Å, a value consistent with other members of this isostructural series.

Direct reaction of Cs and C_{60} has not resulted in superconductivity or the formation of an intercalated f.c.c. phase in our experiments. A recent report¹⁸ of superconductivity at 30 K in Cs_xC_{60} synthesized by reaction of C_{60} with CsM_2 alloys (where M is Hg, Tl or Bi) indicates a T_c lower than we would predict for f.c.c. Cs_3C_{60} . These materials probably adopt a different structure or contain a ternary intercalant. □

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Conversion of methane into higher hydrocarbons on platinum

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CONSIDERABLE effort has been devoted to the conversion of methane into more useful compounds¹. Oxygen has generally been used to draw methane into reaction, at the cost of losing some of the feedstock as carbon dioxide. Several catalytic routes exist for the formation of synthesis gas, $(\text{CO} + \text{H}_2)^{2,3}$, but attempts to synthesize higher hydrocarbons from methane have not yet resulted in a commercially viable process⁴. Here we report that exposure of platinum to pure methane, followed by hydrogenation, can result in appreciable conversion to aliphatic hydrocarbons with up to six carbon atoms. Optimization of this process could lead to a new route to industrially important alkanes.

As the conversion of CH_4 into higher hydrocarbons is thermodynamically unfavourable, partial oxidation has been investigated extensively. In this field most studies have been concerned with oxidative coupling on oxide catalysts at elevated temperature (700–900 °C), resulting in ethane, ethene and carbon monoxide as main interesting products while appreciable total oxidation has not been avoided⁴.

Chain-lengthening reactions involving C_3 or higher alkanes were evidenced, although to a very small extent, over various transition metals^{5,6}. C–C bond formation was also spectroscopically recognized during thermally activated surface rearrangement following adsorption of methyl iodide on $\text{Ru}(001)^7$. Evolution of ethane and even ethene was observed upon adsorption and decomposition of methylchloride on films of several metals⁸. Striking observations were reported concerning the decomposition of diazomethane in the presence of H_2 on transition metals where hydrocarbons similar to those produced in the Fischer-Tropsch reaction were obtained⁹. It has been known for a long time that a number of metals can chemisorb CH_4 and catalyse its exchange with D_2 at moderate temperatures¹⁰. At much higher temperatures and pressures nickel can catalyse the steam reforming of CH_4 . Recently, spectroscopic evidence of the formation of a benzene ring on a $\text{Ni}(111)$ surface through 'collision-induced dissociative chemisorption' followed by adequate heatings has been reported¹¹.

Taking all these observations into account one may imagine that the accumulation of C_1 units on a metal surface through sufficiently long exposure to CH_4 might result in oligomerization processes over adequate metal surfaces.

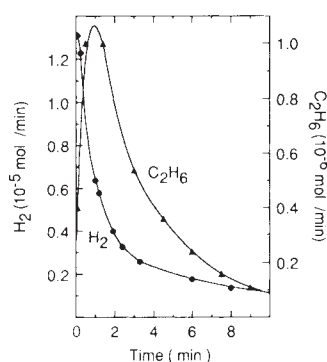


FIG. 1 Molar flow rates of H_2 and C_2H_6 at the exit of the reactor during the exposure of the Pt to CH_4 at 250 °C. Sample mass: 100 mg of EURO PT-1 (see text). Flow rate of CH_4 : 400 ml min^{-1} under ordinary pressure.

The catalyst used in our experiments was a sample of EUROPT-1, which has been a standard platinum catalyst in European catalysis laboratories¹². The catalyst (100 mg) was introduced into a U-shaped quartz reactor, equipped with a disk of fritted silica supporting the sample. The reactor could be fed with flowing CH_4 , H_2 , Ar or He. The four gases were carefully purified and it must be stressed that CH_4 was free of any other hydrocarbon at a detectable level through chromatographic analysis with flame ionization detection (<0.1 p.p.m.).

After reduction of the catalyst by H_2 at 400 °C, followed by a He flush and cooling to the temperature of experiment (150–280 °C), the sample was fed with a flow of pure CH_4 . Figure 1 shows the transient evolutions of H_2 and C_2H_6 that were immediately observed. No other hydrocarbon was produced.

The total amount of C_2H_6 produced since the beginning of the exposure to CH_4 could be roughly estimated to be at any time lower by one order of magnitude than that of H_2 . This means that carbonaceous residues were accumulating over the surface during the exposure, which eventually led to the completion of the chemisorbed film and to the end of the gaseous evolution.

The production of C_2H_6 was not as small as it may appear at first sight if due account is taken of the strong thermodynamic limitation of its formation from CH_4 . At 250 °C and after an exposure of 1 min the rate of the C_2H_6 evolution passed through a maximum and the corresponding quantity $(P_{\text{C}_2\text{H}_6} P_{\text{H}_2}) / P_{\text{CH}_4}^2$ amounted to 40% of the equilibrium constant of the reaction $2\text{CH}_4 \rightleftharpoons \text{C}_2\text{H}_6 + \text{H}_2$.

Fast production of saturated hydrocarbons ranging from C_1 to C_6 or C_7 resulted from the subsequent flush of the solid sample with H_2 at the same temperature. The product composition varied strongly according to the operating conditions. Table 1 gives the amounts of the products other than CH_4 corresponding to an exposure of 1 min to CH_4 at 250 °C.

To know the total amount of CH_4 that was adsorbed during the exposure, the latter was followed in another experiment by temperature-programmed desorption under flowing Ar and temperature-programmed reaction with flowing H_2 of the carbonaceous residues remaining on the surface. To this end, after exposure to CH_4 , the solid sample was quickly cooled to room temperature and the reactor was cleared with a flow of pure Ar. Thereafter the temperature was ramped at 8 K min^{-1} up to 300 °C while the desorption of CH_4 (the only desorbed product) was monitored. The sample was then cooled quickly again to room temperature and Ar was replaced by H_2 . The temperature was ramped in the same way up to 500 °C and CH_4 was the main product thermally reacted from the surface. Under temperature-programmed desorption conditions, not all the CH_x species can leave the surface as they lack hydrogen. The first is stopped at 300 °C to prevent extensive cracking. The remaining species are completely reacted with H_2 during the second run. We could thus determine that the total amount of CH_x residues accumulated over the surface during the 1-min exposure to CH_4 previously referred to, amounted to 8.6×10^{-6} mol. The fraction of CH_4 converted, obviously a function of the operating conditions, was therefore 19.3% in this case. It increased to ~29% when the whole process was conducted at 200 °C but the corresponding amount of higher hydrocarbons produced was lower (1.05×10^{-6} mol) as less CH_4 was adsorbed during the exposure.

These results clearly demonstrate that oligomerization involving CH_x species deriving from CH_4 adsorption can indeed take place. A key factor of success in our experiments was the continuous removal of the H_2 by the flow of CH_4 during exposure. This was checked in performing exposures to CH_4 during equal periods of time but in a closed reactor (batch mode). Only traces of C_2H_6 were obtained. This is easy to understand as the H_2 removal had to be efficient to allow appreciable amount of hydrogen-deficient species to build up on the surface. Formation of C–C bonds can in effect be easily imagined between two neighbouring CH_2 or CH groups. In the

TABLE 1 Example of the amounts and distribution of hydrocarbons formed during the hydrogenation following exposure to CH₄

	C ₂	C ₃	<i>i</i> -C ₄	<i>n</i> -C ₄	<i>i</i> -C ₅	<i>n</i> -C ₅	cyclo C ₅	<i>n</i> -C ₆	other C ₆	Total CH ₄ converted
Amount (10 ⁻⁸ mol)	52.8	7.11	0.25	3.19	0.10	2.49	1.74	0.34	0.32	166.3
Selectivity (%)	63.5	12.8	0.60	7.69	0.30	7.50	5.24	1.23	1.16	—

Conditions: 100 mg of EURO PT-1. (a) Step 1: 1-min exposure of the catalyst to a flow of CH₄ (400 ml min⁻¹; 1 atm; 250 °C). (b) Step 2: hydrogenation of the adsorbed species by a flow of H₂ (50 ml min⁻¹; 1 atm; 250 °C). The selectivity of each hydrocarbon is defined as the ratio of the molar amount of CH₄ converted into this hydrocarbon to the total molar amount of CH₄ converted.

state so obtained the chemisorbed film can be viewed very much like the one resulting from adsorption of alkenes or alkynes. But spontaneous desorption of unsaturated products was not observed at the temperatures of the experiments and heating at higher temperatures was avoided in order not to cause extensive cracking. On the contrary, submitting the carbonaceous species to a flow of H₂ entailed their hydrogenation and the desorption of the resulting alkanes, which revealed the occurrence of chain-lengthening processes.

This analysis was confirmed by an additional experiment consisting of feeding the sample with a flow of CO just after the exposure to CH₄ and before that to H₂. Most of the gaseous production in this case was observed under CO and a large part of the products were unsaturated hydrocarbons ranging from C₂ to C₇ which were dislodged from the surface by CO. More information will be given in future reports.

To explain why similar results were not obtained in preceding

work when carbonaceous residues resulting from chemisorption of CH₄ were subjected to the action of H₂, we propose one or several of the following reasons. (1) The metals chosen were not good candidates; (2) the temperature was too low to allow sufficient CH₄ adsorption and/or H₂ desorption; and (3) the use of a closed reactor did not allow sufficient dehydrogenation to be effected during adsorption.

Two significant advantages of the procedure suggested here for converting CH₄ into higher hydrocarbons are that the temperatures required are much lower than those needed for oxidative coupling and that no methane need be wasted, as that which is not converted can be recycled. But the low conversion efficiency in each cycle is presently a limitation. Contamination of the catalyst surface by deposits of carbon is likely after many cycles; nevertheless, we observed no deactivation after ten successive cycles at 250 °C. We are currently studying these aspects in more detail. □

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Reconstruction of Caribbean climate change over the past 10,500 years

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SEDIMENT cores from low-latitude lakes provide some of the best records of tropical climate change since the late Pleistocene. Here we report a high-resolution reconstruction of Caribbean climate based on ¹⁸O/¹⁶O ratios in ostracod shells from Lake Miragoane, Haiti. Our results show that the climate was dry and the lake level low during the latter part of the Younger Dryas chronozone (10.5–10 kyr BP), but that water level in the lake rose at the end of the last deglaciation (~10–7 kyr BP), reflecting the wetter conditions of the early Holocene which persisted for nearly 4,000 years. Lake level declined at ~3.2 kyr BP with the onset of a drier

climate which generally prevailed throughout the late Holocene. These long-term changes in Caribbean climate are generally similar to those found for Africa over the same period, and can be largely explained by orbitally induced (Milankovitch) variations in seasonal insolation which modified the intensity of the annual cycle. Superimposed on the orbitally forced climate trends are more abrupt climate events that result from complex, nonlinear interactions in the ocean–atmosphere system. The interpretation of Antillean biogeography and the development of Caribbean and Mesoamerican culture must be considered in the context of such shifts in climate.

Lake Miragoane (area 7.06 km², maximum depth 42 m) lies on the north side of Haiti's southern peninsula (Fig. 1). It lies ~5 km from the coast and its surface is 20 m above mean sea level. The residence time of water in the lake is estimated to be about three years¹. We measured oxygen isotopes on mono-specific samples of fossil ostracods (*Candona* sp.) from a 7.7-m core taken from the lake's centre. The core was sampled at 1-cm intervals corresponding to an average sample spacing of ~20 years. High-resolution data, in conjunction with a precise radiocarbon chronology (Table 1), permit detailed interpretation of Holocene climate change from this tropical Atlantic locality.

Today, Lake Miragoane is not a closed basin because it has a small outlet on its northern shore, but outflow across this shallow sill did not occur during times of lowered lake level. In closed-basin lakes with seasonally dry climate, the ¹⁸O/¹⁶O ratio of lake water is controlled mainly by the ratio of evaporation to precipitation^{2,3}. Extended periods of high evaporation lower the lake levels and raise the ¹⁸O/¹⁶O ratios. Here we assume that variations in the oxygen isotopic composition of shell calcite of *Candona* sp. reflect changes in the ¹⁸O/¹⁶O ratio in lake water.

TABLE 1 Radiocarbon results of organic and inorganic carbon from a core from Lake Miragoane, Haiti.

Core depth (m)	Type/description	Target number	Accession number	Measured age (yr)	Corrected age (yr)
0.08	^{210}Pb			129 ± 40	29
0.22	AMS ^{14}C /ostracods	WHG908	AA6703	1,085 ± 60	85
2.16	AMS ^{14}C /ostracods	WHG775	AA5814	2,780 ± 55	1,780
2.33	AMS ^{14}C /ostracods	WHG909	AA6704	2,680 ± 60	1,680
2.33	AMS ^{14}C /wood	WHG917	AA6705	1,655 ± 60	1,655
3.21	AMS ^{14}C /ostracods	WHG777	AA5815	4,110 ± 60	3,110
4.18	AMS ^{14}C /ostracods	WHG776	AA5816	4,780 ± 60	3,780
5.20	AMS ^{14}C /ostracods	WHG782	AA5817	6,945 ± 65	5,945
6.22	AMS ^{14}C /ostracods	WHG784	AA5818	9,005 ± 75	8,005
6.71	AMS ^{14}C /ostracods	WHG720	AA5369	9,700 ± 90	8,700
7.18	AMS ^{14}C /ostracods	WHG785	AA5952	10,300 ± 85	9,300
7.53	Conventional ^{14}C /bulk organic carbon		GX13055	10,230 ± 160	10,230

All AMS samples were converted to graphite at Woods Hole Oceanographic Institution²⁹ and measured at the University of Arizona. Because Lake Miragoane is a hard-water lake, radiocarbon dates are susceptible to error resulting from the contribution of bicarbonate derived from the dissolution of 'old' carbonate rock (devoid of ^{14}C)³⁰. To estimate this effect, we measured a paired wood and ostracod sample from 2.33 m which gave an age difference of 1,025 years. Assuming a constant hard-water error throughout the core, we subtracted 1,000 years from the ostracod dates to obtain corrected ages. This correction yields good agreement between ^{210}Pb , organic ^{14}C and carbonate ^{14}C dates at all levels. The resultant age-depth pairs are well described by a second-order polynomial equation of the form: Age (yr BP) = 47.2 + 394.8 d + 127.2 d^2 , where d is the depth in metres.

Because Lake Miragoane is situated on an island whose climate is strongly influenced by the ocean, changes in its water budget should reflect large-scale processes in the ocean-atmosphere system.

The oxygen isotope data from Lake Miragoane were filtered using a five-point running mean to smooth out variations on timescales of less than 100 years. The highest $\delta^{18}\text{O}$ values are found near the base of the core, between ~10.5 and 10 kyr, indicating drier climate, increased evaporation and lowered lake level (Fig. 2). The inference for an arid climate is supported by palynological data indicating open, dry vegetation characterized by xeric palms and montane shrubs (zone P1, Table 2)⁴. This interval falls in the latter part of the Younger Dryas chronozone (11–10 kyr BP⁵; ref. 5), which marked a brief return to cold conditions in Europe and North America during the middle of the last deglaciation. Studies of North African lakes also demonstrate increased aridity and lowered lake levels during the Younger Dryas, with maximum aridity recorded between 10.6 and 9.3 kyr BP in the northern Sahara and between 10.3 and 10.0 in the Sahel^{2,6}.

From ~10.0 to 7.0 kyr BP, a general trend toward decreasing $\delta^{18}\text{O}$ values indicates a change from low to high water levels

(Fig. 2). This transition was not smooth, however, and at least two abrupt minima in $\delta^{18}\text{O}$ occurred at ~9.1 and 8.1 kyr BP. This trend marks a switch from arid to humid conditions, which has been documented throughout much of the tropics near the Pleistocene/Holocene boundary^{2,6–15}. The interval coincides with the second step of the last deglaciation (termination IB) in marine oxygen isotope records^{16–18}. Lake Miragoane's increased water level during the early Holocene is attributed to greater precipitation as well as to a rising sea level which raised the phreatic aquifer. Although water level was generally rising during the early Holocene, the ratio of evaporation to precipitation was still high between ~10 and 8.4 kyr BP, as indicated by relatively high $\delta^{18}\text{O}$ values (Fig. 2). The pollen assemblage also indicates widespread dry conditions until ~8.2 kyr BP, when a sharp increase in *Chenopodiaceae*/*Amaranthaceae* pollen suggests a rising lake level and the development of an extensive littoral zone⁴ (P1/P2 boundary; Table 2). This shift in the pollen assemblage coincides with a sharp decrease in $\delta^{18}\text{O}$, indicating an abrupt decrease in the ratio of evaporation to precipitation (Fig. 2).

In Lake Miragoane, lake levels were high between ~7.0 and 5.3 kyr BP, as indicated by the lowest $\delta^{18}\text{O}$ values of the record

FIG. 1 Map of the Caribbean region indicating the palaeoecological study sites discussed in the text (black dots). Numbers at each site are the reference numbers for the following circum-Caribbean localities: Lake Annie, Florida, USA¹⁵; Lakes Salpeten and Quexil, Guatemala^{8,10,19}; Lake La Yeguada, Panama¹²; Lake Miragoane, Haiti^{1,4} (this study). The arrow indicates the location of Lake Miragoane on the north side of Haiti's southern peninsula.

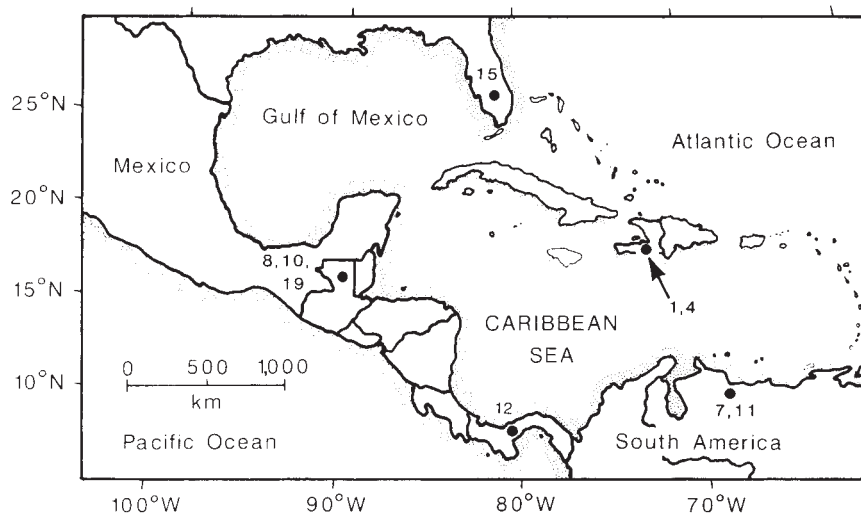


TABLE 2 Pollen zonation of Lake Miragoane core with lake level and climatic interpretations.

Age yr BP	Pollen Zones	Vegetational changes ⁴	Lake level	Climate
~1,000	P5	Considerable loss of mesic Forest trees (for example Moraceae); Vegetation dominated by dry forest taxa and abundant weeds	Rapidly declining	Dry
2,500	P4	Mature mesic forest with Moraceae dominant		
3,900	P3	Pioneer mesic forest represented by <i>Trema</i> and <i>Cecropia</i>	High	Increasingly mesic
5,400	P2	Moderate forest growth; Chenopodiaceae/ Amaranthaceae abundant in littoral zone	Rapidly rising	
8,200	P1	Non arboreal communities dominated by xeric palms and montane shrubs	Lower than today	Arid
>10,400				

Ages for boundaries between pollen zones are based upon the equation in Table 1. Low and high lake level stages, inferred from oxygen isotope variations, generally coincide with episodes of dry and mesic vegetation, respectively. No climatic interpretations of pollen after 1,000 yr BP (zone P6) were made because of possible human disturbance of vegetation.

(Fig. 2). This interval corresponds to the early to mid Holocene moist period when lake levels were higher than today throughout much of the northern American tropics^{7,8,10-12} and Africa^{14,20,21}. Evaporation increased slightly relative to precipitation at 5.2 kyr BP, as indicated by a small increase in mean $\delta^{18}\text{O}$ (Fig. 2). Lake levels remained relatively high between 5.2 and 3.2 kyr BP. This deep-water phase of the lake coincided with the development of mesic forests in Miragoane's watershed during the middle Holocene⁴ (Table 2).

Between 3.2 and 2.4 kyr BP, a two-step increase in $\delta^{18}\text{O}$ indicates a trend towards higher evaporation rates and lower lake level (Fig. 2). The sharp increase at 2.4 kyr BP marks the beginning of a dry episode that lasted from ~2.4 to 1.5 kyr BP. Further indications of a return to dry conditions are a considerable decline in mesic forest taxa (such as Moraceae, *Cecropia* and *Trema*) and the establishment of open, dry forest communities associated with abundant weeds (zone P5; Table 2)⁴. A brief period of wetter conditions recurred between ~1.5 and 0.9 kyr BP, and was followed by a progressive increase in the ratio of evaporation to precipitation until the present (Fig. 2).

The overall climate pattern, involving dry conditions during the Younger Dryas chronozone (11 to 10 radiocarbon kyr BP), followed by a wetter early to mid Holocene, and a return to drier conditions during the late Holocene is similar to the pattern reported for Africa^{2,6,9,13-14,20-21} and the region around the Caribbean^{7-8,10-12,19}. The history of individual fluctuations in lake levels may differ in detail, however, because of regional or local controls on hydrological budgets. Because the general pattern of lake-level history is similar for these two tropical regions, we suspect a common driving mechanism.

In the Caribbean region, the rainy season occurs during the northern summer half-year when the intertropical convergence zone moves north, displacing the North Atlantic subtropical high and weakening the easterly trade winds^{22,23}. Meteorological studies of interannual variability of rainfall in the tropical Atlantic sector during the twentieth century have found a strong correlation between precipitation anomalies and intensity of the annual cycle²³. Enhancement of the annual cycle led to years

of anomalously high precipitation, whereas a reduction led to a deficient rainy season²³. Following this lead, we compared the $\delta^{18}\text{O}$ record from Lake Miragoane with changes in the intensity of the annual cycle as estimated by the seasonal insolation difference at the top of the atmosphere at 10° N between August and February (Fig. 2). The long-term changes in the two records are similar, suggesting that changes in the evaporation/precipitation ratio are controlled generally by orbitally forced (Milankovitch) variations in solar insolation which modify the intensity of the annual cycle in the Caribbean. Similarly, lake-level fluctuations in east Africa have also been correlated with the intensity of the annual cycle because of its effect on the monsoons and rainfall over Africa^{7,21,24-27}.

Direct orbital forcing alone, however, does not fully explain the magnitude or rate of water-level change recorded in lake Miragoane⁶. For example, inferred aridity at the close of the last glaciation (from 10.5 to 8.4 kyr BP) and during the late Holocene (from 2.4 and 1.5 kyr BP) is more pronounced than predicted by insolation forcing (Fig. 2). Furthermore, the slow changes in insolation forcing do not account for some of the abrupt shifts observed in the $\delta^{18}\text{O}$ record, such as the sudden onset of dry conditions at 3.2 kyr BP (Fig. 2). Identifying the mechanisms responsible for rapid climatic shifts during the Holocene will be a priority for future palaeoclimatic research.

The climate changes recorded in isotopic and pollen data from Lake Miragoane may have important implications for the distribution of West Indian fauna as well as regional cultural

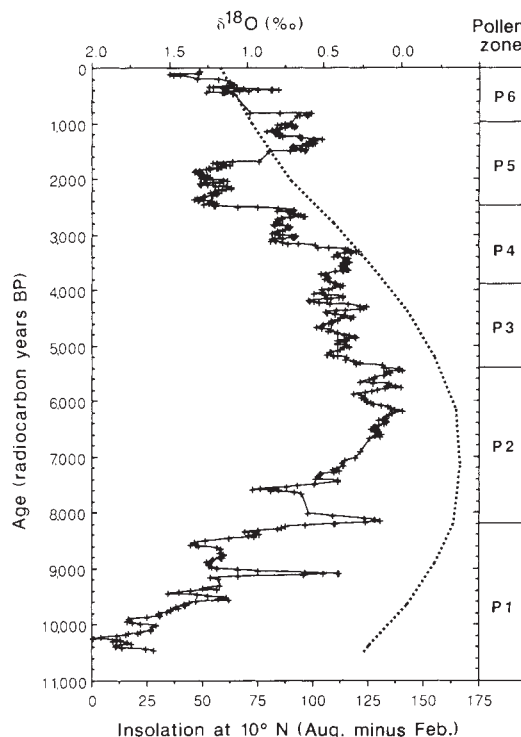


FIG. 2 Oxygen isotope measurements (relative to the PDB reference) of a single species of ostracod (*Candona* sp.⁶) from a 7.7-m core taken in 41 m of water from Lake Miragoane, Haiti. $\delta^{18}\text{O} = \{[(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{ref}}] - 1\} \times 1000$. The ratio of evaporation to precipitation is high to the left of the figure, low to the right. Ages for isotopic samples were derived using the age-depth model given in Table 1. The isotopic data were filtered using a five-point running mean, which averages ~100 years over most of the core. Explanation of pollen zonation is provided in Table 2. The dotted line represents the difference in insolation (Langleys) at the top of the atmosphere at 10° N latitude between the months of August and February³¹, which is a measure of the intensity of the annual cycle. Sidereal ages for insolation values were converted to radiocarbon years^{32,33} for comparison with the oxygen isotope record.

development during the Holocene. For instance, climatic shifts since the late Pleistocene may have played a part in causing high rates of mammal extinctions reported for the West Indies²⁸. Furthermore, abrupt climate fluctuations during the late Holocene may have influenced the development of prehistoric cultures in the Caribbean and nearby Mesoamerica. Testing the regional significance of the climate changes outlined here will require similar high-resolution studies in other lowland Neotropical lakes. □

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Dispersion and advection in unsaturated porous media enhanced by anion exclusion

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It has been observed^{1–4} that the average transport velocity of dissolved anions through soils may be larger than that of the accompanying water molecules, owing to electrostatic repulsion by negatively charged solid surfaces, which forces the anions into pore centres where the velocity is faster. This phenomenon, known as anion exclusion, has been explained by diffusive double-layer theory^{5–7}. Here we present analyses and numerical modelling of concentration/depth profiles of tritium, chloride and sulphate which were collected from irrigated land in the Israeli coastal plain. We found that the anions travelled at about twice the velocity of tritium. The behaviour of tritium is consistent with advective–diffusive transport, but the values of the dispersion coefficients

associated with anion transport greatly exceeded the values expected for molecular diffusion in a porous medium, and were ~30 times those found for tritium transport. Our results indicate that anion exclusion restricts the number of active pore networks available for anion transport. We present two conceptual models that can explain the observed results—in one model some porous regions are completely blocked, whereas in the other they are only partially blocked.

The phenomenon of anion exclusion has been demonstrated in experimental studies^{1–4} and has been explained by diffusive double-layer theory^{5–7}. As a result of anion exclusion, the average transport velocity of dissolved anions through soils may be larger than that of the accompanying water molecules because of electrostatic repulsion by negatively charged solid surfaces which forces the anions into the pore centres where the velocity is faster. As recognized here, exclusion may greatly reduce the amplitude between extreme values in the anion concentration profiles, which can be described by enhanced dispersion.

During a field investigation of an irrigated region, 10 km north of Tel-Aviv⁸, solute migration was studied. Precipitation and irrigation each had characteristic chemical source-signatures. The 27-yr chronological record of source events is imprinted in the depth profile of solute concentration in the sediment. Concentration peaks and minima corresponding to winter rains and summer irrigation were identified and dated. The vertical profile consists of 27.5 m of water-unsaturated sediments. Three horizons characterize the profile: (1) the upper 0.9-m soil unit, (2) a 9-m-thick clay loam layer and (3) an underlying homogeneous sandy layer that extends down to the water table. The boundary between each zone is characterized by an abrupt change in the unsaturated water content. The influence of the capillary fringe extended ~2.5 m above the water table.

Figure 1 shows solute data consisting of concentrations of tritium, chloride and sulphate that were measured in the extracted solution of samples collected along the profile. Atmospheric thermonuclear tests during the 1950s and 1960s caused rainwater to become enriched in tritium. This rainwater then infiltrated the soil. The history of the tritium concentrations in rain is known in this region⁸ as it is in many locations throughout the world⁹. Isotopic enrichment of tritium due to transpiration is negligible¹⁰. The source of chloride was irrigation water derived from sewage effluent high in chloride. Sulphate was a component of ammonium sulphate fertilizer. The average recharge of 150 mm yr⁻¹ is 17% of the total rate of applied water. The rest is evapotranspired. Seasonal wetting and drying affects the upper soil, but can be neglected below 2 m where steady-flow conditions exist.

The peaks in the three concentration profiles were correlated with their seasonal chronological input events. From the vertical separation of the peaks and initial simulations, we determined the average vertical velocities through the lithologic zones (Table 1).

The three sets of concentration data were successfully reproduced using two different conceptual models. First is the 'dispersion model' which involves transient one-dimensional advective–dispersive transport under steady-flow conditions. For tritium, first-order kinetic decay was included. To represent the effects of anion exclusion, anion transport was divided into two regimes: an excluded volume near the solid surfaces containing no anions, and a mobile volume in which all of the anions are concentrated. The general governing equation³ is

$$(\theta - \theta_{ex}) \frac{\partial C}{\partial t} = \frac{\partial}{\partial z} \left[(\theta - \theta_{ex}) D_h \frac{\partial C}{\partial z} \right] - Q \frac{\partial C}{\partial z} - (\theta - \theta_{ex}) \lambda C \quad (1)$$

where θ is the unsaturated water content (dimensions L³/L³); θ_{ex} is the water content of the excluded volume (L³/L³) (θ_{ex} is zero for tritium, and the water content of the mobile domain is $\theta_m = \theta - \theta_{ex}$); C is the solute concentration (M/L³); D_h is the hydrodynamic dispersion coefficient, $D_h(z)$ (L²/T); Q is the

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TABLE 1 Transport parameter values for clay-loam zone

Solute	Dispersion model				Exchange model						
	E_i	θ_m	θ_{ex}	V (cm d ⁻¹)	D_h (cm ² d ⁻¹)	D_a (cm ² d ⁻¹)	D_p (cm ² d ⁻¹)	V' (cm d ⁻¹)	θ_{ex}	θ_{im}	α (1/d)
³ H	1.00	0.190†	0.000	0.202‡	0.072 ± 0.01§	1.469¶	0.072#	0.202	0.000††	0.000	—
Cl ⁻	0.56*	0.106	0.084	0.360‡	1.67 ± 0.25§	1.296¶	0.036**	0.439††	0.061‡‡	0.023 ± 0.004§	0.0034 ± 0.0012§
SO ₄ ²⁻	0.46*	0.087	0.103	0.440‡	2.93 ± 0.95§	0.657¶	0.015**	0.537††	0.084‡‡	0.019 ± 0.005§	0.0024 ± 0.0013§

* Mobile fraction = velocity of tritium/velocity of anion.

† For tritium, the total water content is mobile.

‡ Based on peak separation, Fig. 1.

§ Calibrated value and standard deviation of estimate.

¶ Taken at 13 °C (ref. 21).

¶ Taken at 13 °C (ref. 22).

Calibrated value D_h from dispersion model.

** Microscale tortuosity, 0.05, calculated using equation (2) for tritium and applied to anions as dispersion coefficient in exchange model.

†† Actual anion average velocity, V' , is the product of the apparent velocity, V , based on peak separation and $(1 + \theta_{im}/\theta_m)$.

‡‡ $\theta_{ex} = \theta - \theta_m - \theta_{im}$.

darcian flux (L/T) related to the average velocity, V , by $V = Q/(\theta - \theta_{ex})$; λ is the first-order kinetic decay rate for tritium (1/T); t is time (T); and z is vertical distance (L) along the flow direction.

For average velocities of tritium seen here, mechanical dispersion is negligible with respect to molecular diffusion^{11,12}. The molecular diffusion coefficient in porous media, $D_p(L^2/T)$, is related to the diffusion coefficient in a free fluid, $D_d(L^2/T)$, by

$$D_p = D_d \tau E_i \quad (2)$$

where τ (where $\tau < 1.0$) is tortuosity¹³, and E_i is the fraction of the unsaturated water content associated with transport of anion i ($E_i = 1.0$ for tritium). The mobile fraction, $E_i = \theta_m/\theta = V_{\text{tritium}}/V_{\text{anion}}$, is obtained from the relationship, $V_{\text{anion}}\theta_m = V_{\text{tritium}}\theta$.

Second is the 'exchange model', which involves advective-diffusive transport of anions in the mobile domain plus diffusional exchange with an immobile domain. The water content of the immobile domain is θ_{im} , and the excluded portion is θ_{ex} . The total unsaturated water content is now defined as

$$\theta = \theta_{ex} + \theta_m + \theta_{im} \quad (3)$$

The coupled governing equations for the mobile and immobile domains are

$$\theta_m \frac{\partial C_m}{\partial t} = \frac{\partial}{\partial z} \left[\theta_m D_p \frac{\partial C_m}{\partial z} \right] - Q \frac{\partial C_m}{\partial z} - \theta_m \alpha (C_m - C_{im}) \quad (4)$$

$$\theta_{im} \frac{\partial C_{im}}{\partial t} = \theta_m \alpha (C_m - C_{im}) \quad (5)$$

where C_m and C_{im} are the anion concentrations in the mobile

and immobile domains; and α is the mass transfer rate coefficient between the mobile and immobile domains (1/T).

The governing equations were solved numerically using the finite element method with 48,000 spatial nodes and 200 time steps per year. Inflow was modelled using a series of 54 seasonal solute flux values, corresponding to alternating winters and summers during the years 1957–84. The duration of each season was adjusted during calibration but was tightly limited because any single change in the duration of a winter or summer affected all three independently simulated profiles. Values below 25 m, influenced by the capillary fringe, were disregarded. Calibration employed simulation combined with nonlinear extreme-value weighted least-squares regression¹⁴.

Figure 1a–c shows the calibrated simulations using the 'dispersion model' and the field data for tritium, chloride, and sulphate concentrations along the profile after 27 yr of transport (1957–84). The values of the dispersion coefficients were calibrated in the clay-loam and fixed in the sand at their values of molecular diffusion in porous media (Table 1). In the sand, tritium and anions had the same velocity. In the clay-loam, however, the average velocity of the three constituents increased in the order tritium to chloride to sulphate. The greater the charge, the greater the repulsive forces from the charged surfaces. The D_h value for tritium in both sediment horizons and the values of D_h for the anions in the sand are consistent with values of molecular diffusion in porous media (Table 1). The D_h values for the anions in the clay-loam layer, however, were 23 and 41 times that of tritium. Sensitivity analyses, using the D_h value of tritium to simulate anion transport, doubled the range of concentration values along the profile compared with the anion field data.

FIG. 1 Comparisons of simulated concentrations with field data for a, tritium, b, chloride and c, sulphate using the 'dispersion model', and for d, chloride and e, sulphate using the 'exchange model'. Simulations a–c were based on a finite-element solution to the governing advective-dispersive equation, whereas d and e were based on the finite-element solution to the coupled mobile-immobile domain transport equations. Parameter values associated with the clay-loam layer for the two simulation models are shown in Table 1. In the sand/sandstone unit the solute velocity for all three constituents was 0.50 cm d⁻¹, and the hydrodynamic dispersion coefficient was 0.05 cm² d⁻¹.

METHODS. Tritium was measured by low-level β -counting and values are given in tritium units (TU) where 1 TU corresponds to 1 atom per 10¹⁸ hydrogen atoms. Chloride and sulphate were measured by ion chromatography using high-pressure liquid chromatography⁸. Gravimetric water content was measured by weighing the sample before and after drying. Volumetric water content was calculated using bulk density values of 1.55 and 1.28 g cm⁻³ for clay loam and sand, respectively.

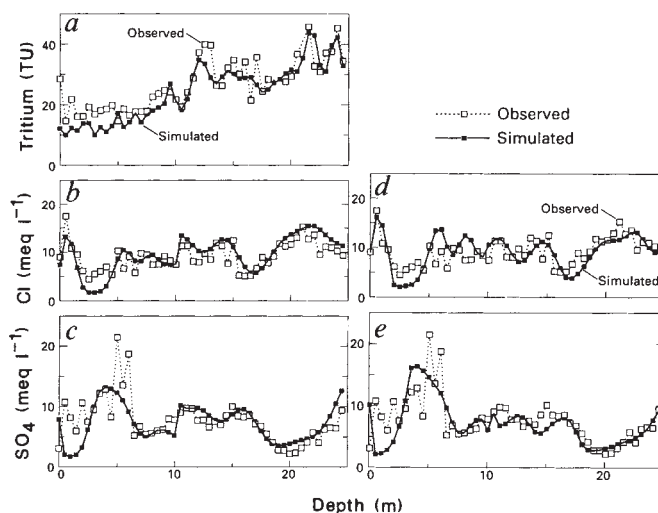


Figure 1d, e shows the calibrated simulation results using the 'exchange model' (Table 1). This model was used only to simulate anion transport. Tritium transport was described using the 'dispersion model'. The dispersion coefficients were fixed at their values of molecular diffusion, D_p (Table 1). Diffusional exchange reduces the advective velocity because the anions spend some time in the immobile domain. Anion velocity was related to the fractional water content of the immobile versus mobile domains (Table 1). Calibrated parameters for the 'exchange model' are θ_{im} and α . Compared with the monovalent ion, exclusion of the divalent ion may generate smaller immobile pockets (smaller θ_{im}) with narrower passages through which diffusive exchange with the mobile domain occurs (smaller rate coefficient, α).

The value of the apparent dispersion coefficient used in the 'dispersion model' was calculated to within 10% of the parameter estimate when using¹⁵

$$D_h = \frac{\theta_m}{\theta_m + \theta_{im}} \left[D_p + \frac{V^2}{\alpha} \left(\frac{\theta_{im}}{\theta_m + \theta_{im}} \right)^2 \right] \quad (6)$$

with the values of parameters in the 'exchange model'.

The effect we document shows that, compared with tritium transport, anion advection is doubled, and attenuation of the anion concentration profiles is greatly enhanced. Both effects are believed to be caused by anion exclusion.

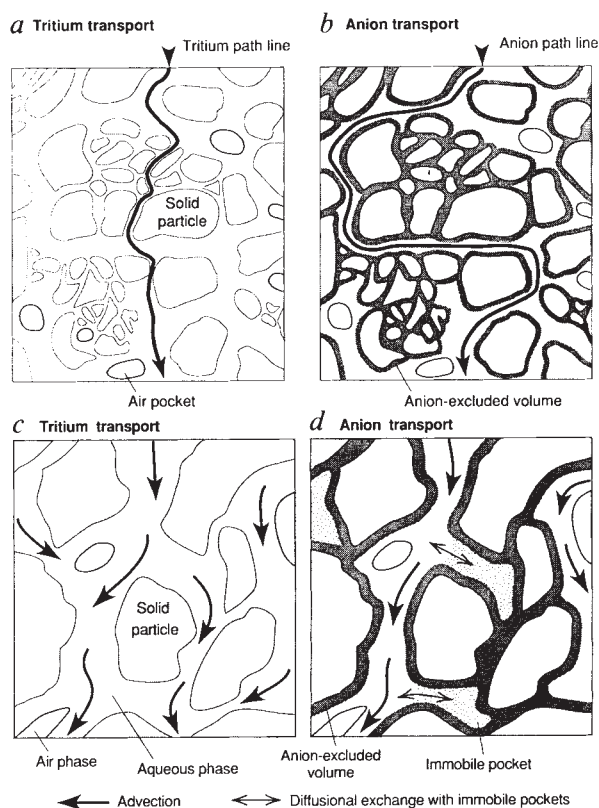


FIG. 2 Schematic model of pore-scale flow networks controlling migration according to the 'dispersion model', a, b, and the 'exchange model', c, d. Electrostatic forces repel anions from negatively charged surfaces, thereby excluding them from the small pores of the unsaturated porous medium. In the 'dispersion model' anions necessarily follow a more tortuous pathway compared with that of tritium, which is not subject to ion exclusion. Dispersion coefficient values associated with the transport of anions are much greater than that of tritium, perhaps, because anions 'see' an effectively more heterogeneous porous medium. In the 'exchange model' anion exclusion creates isolated dead-end pockets which are connected to the mobile domain by larger pore passageways through which diffusional exchange occurs. Tritium is not excluded from the small pores and flows freely through the entire network.

The 'dispersion model' generates attenuation by permitting increased mechanical dispersion. Values of dispersion coefficients for chloride and sulphate exceed their values of molecular diffusion coefficients in the porous medium by 46 and 195 times, respectively. Yet tritium transport behaviour was successfully reproduced by a model of molecular diffusion alone. It is evident that anion transport must involve some means of attenuating extreme values of concentration that cannot solely be caused by molecular diffusion.

Perhaps anion exclusion creates enhanced dispersion by generating heterogeneities that serve as flow barriers. Figure 2a, b shows a single porous medium and compares the active flow network for tritium with that for anions. Tritium is able to flow through both large and small pores. On the other hand, anions are excluded from small pores, which may be concentrated in aggregates. Anion flow, which is restricted to the larger pores, will be more tortuous than that of tritium because of the aggregated obstacles. The larger dispersion coefficients for anions may reflect this additional velocity variation due to heterogeneity¹⁶⁻¹⁸.

An alternative explanation employs the 'exchange model' which generates attenuation by allowing for immobile zones that are created during anion transport. Figure 2c, d compares the flow network for tritium with that for anions. Tritium flow is unrestricted, whereas anions are excluded from small pores, thereby creating nearly isolated immobile pockets that are connected to the mobile zone by larger pores. Mass transfer between the mobile and immobile zones attenuates extreme values of concentration. Using this model, an immobile fraction occupying about one-tenth of the total water content best reproduced the concentration data. The values of the mass transfer coefficient were found to be two to three orders of magnitude less than those reported from laboratory studies^{15,19,20}. Model results indicate that compared with the monovalent ion (chloride), the divalent anion (sulphate), has a greater exclusion volume surrounding the clay surfaces, smaller dead-end pockets, and smaller passageways through which mass transfer occurs.

Of most significance is the fundamental similarity between the two conceptual models. Both models maintain that anion exclusion greatly influences the active porous network. Because anions cannot flow through small pores, some porous regions may be completely blocked ('dispersion model') or partially blocked ('exchange model'). The geometry of blockages is the significant difference between the two models. For both conceptual models there are restricted active flow networks experienced by anions during transport which enhance the attenuation of extreme values in the concentration profiles. □

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Pollen vigour and the potential for sexual selection in plants

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DISCOVERING whether paternal fecundity varies within populations is crucial for evaluating whether male-male competition and/or female choice can lead to sexual selection¹⁻³. In natural populations of plants, pollinators often deposit mixtures of 'surplus' pollen from several individuals onto receptive stigmas⁴⁻⁷. Therefore within-flower competition among pollen-tubes for ovules could lead to nonrandom paternal success. We found that pollen competition was common in populations of wild rose mallow (*Hibiscus moscheutos*). Co-occurring individuals often differed in mean pollen-tube growth rates, and this trait was correlated with the number of seeds the plants sired when pollen mixtures were applied to stigmas. Differences between pairs of individuals in pollen-tube growth rates were consistent across maternal plants, suggesting that sexual selection can occur. This unexpected variation in pollen vigour could lead to nonrandom fertilization whenever pollen-tubes compete for access to ovules.

Hibiscus moscheutos is a perennial marsh plant that is self-compatible but predominantly outcrossing⁸. At our study site in Maryland, the large, one-day flowers were frequently visited by bumble and anthophorid bees. Flowers opened synchronously at about 0600 h, 1-2 h before pollinator activity. By mid-morning, bees typically delivered more pollen than is needed for full seed set, and later in the day the ratio of deposited pollen grains to ovules often exceeded 8:1 (T.P.S. and A.A.S., manuscript submitted). As with many other species, it is likely that pollen-tubes from different individuals often compete for access to a limited number of available ovules⁷.

To determine whether individuals vary in average pollen-tube growth rates, we collected a random sample of 60 genotypes from a natural population at the Smithsonian Environmental Research Center, Edgewater, Maryland. These plants were grown in large pots in an outdoor garden near the original population. In 1989 the plants were used to compare the performance of pollen from six randomly chosen pairs of pollen donors across three to five recipients per pair (Table 1). We quantified pollen-tube growth rates by applying pollen from each donor in a pair to a separate stigmatic branch of the same flower. Relative rates of pollen germination and pollen-tube development were measured in styles collected after 3 h (most pollen germinates in 1 h; methods are described in ref. 9).

Average pollen-tube growth rates were unequal in four of the six donor pairs tested in 1989 (Table 1). Because pollen-tube growth can be influenced by the stylar environment⁷, we tested for maternal effects on relative pollen performance. Statistically, this interaction was not significant (Table 1), although maternal effects may be real. As shown in Fig. 1, for example, the magnitude of differences between pollen donors varied across recipients. It is clear, however, that the ranking of donors (as 'fast' versus 'slow') did not change.

In 1990 we performed a similar experiment in which three pairs of pollen donors were compared on one flower from each of 14-15 unrelated plants (Fig. 2). Four of the donors had been used in 1989 and were known to vary in pollen-tube growth rates; these donors were also tested for differences within recipients (Table 1). Again, we found significant differences between donors, confirming previous results and further demonstrating that pollen competitive ability was consistent across a larger sample of recipient genotypes. Previously we used a

TABLE 1 Comparisons of pollen germination and pollen-tube growth rate for pairs of pollen donors across recipients

Pollen donor ID A versus B	Recipient plant ID (Year)	Pollen germination Mean number of tubes per pollen grain A versus B		Pollen-tube growth Mean number of callose plugs per tube A versus B		N
17 versus 26	(1989)					
	4	0.62	0.58	0.38	0.31	8
	32	0.61	0.58	0.22	0.21	12
32 versus 33	(1989)					
	33	0.66	0.56	0.27	0.23	11
	17	0.61	0.65	0.31	0.28	12
37 versus 41	(1989)					
	26	0.75	0.71	0.29	0.37	15
	36	0.71	0.61	0.28	0.38	10
53 versus 51	(1989)					
	1	0.67	0.69	0.29	0.26	14
	5	0.73	0.63	0.39	0.33	14
54 versus 60	(1989)					
	38	0.68	0.61	0.37	0.27	14
	54	0.65	0.60	0.46	0.35*	19
45 versus 63	(1989)					
	50	0.78	0.64	0.38	0.31*	16
	23	0.66	0.68	0.27	0.32	12
53 versus 60	(1989)					
	27	0.69	0.68	0.40	0.23**	12
	45	0.78	0.86	0.38	0.20**	16
54 versus 60	(1989)					
	63	0.71	0.70	0.29	0.23	15
	6	0.78	0.75	0.37	0.22**	16
45 versus 63	(1989)					
	20	0.64	0.67	0.31	0.29*	16
	28	0.62	0.69*	0.45	0.33**	15
53 versus 60	(1989)					
	37	0.70	0.66	0.63	0.37***	15
	41	0.79	0.78	0.53	0.36†	10
53 versus 60	(1989)					
	39	0.72	0.67	0.26	0.18	11
	46	0.80	0.59*	0.27	0.09*	10
53 versus 60	(1990)					
	51	0.75	0.77	0.41	0.26*	15
	53	0.72	0.72	0.40	0.26†	15
53 versus 60	(1990)					
	21	0.92	0.82	0.59	0.41***	11
	46	0.87	0.90	0.53	0.30***	11
53 versus 60	(1990)					
	53	0.89	0.85	0.58	0.43**	16
	59	0.92	0.82*	0.60	0.44*	8
53 versus 60	(1990)					
	1	0.86	0.77	0.62	0.39***	20
	6	0.90	0.88	0.59	0.36***	11
53 versus 60	(1990)					
	20	0.91	0.79	0.61	0.41***	14
	28	0.85	0.88	0.65	0.44***	17

Pollen grains were counted on fresh stigmas. Pollen-tubes from pairs of individuals were counted using fluorescence microscopy, and the number of callose plugs present in a standardized cross-sectional band in the mid-stylar region was determined⁹. The number of callose plugs per tube is a sensitive indicator of relative pollen-tube growth rate⁹. Because comparisons between donors were planned, paired *t*-tests were used to compare means; significance levels are † $P < 0.06$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (faster donor is underlined). Differences between donors in per cent pollen germination were rarely significant; hence we conclude that this effect on male fitness is much less important than differences in pollen-tube growth rate. When significant differences in the latter were detected, ANOVAs were performed using arcsin-transformed proportions and the general linear models procedure of SAS (ref. 28). Sources of variation were donor, recipient, their interaction, and flowers nested within recipients. Pollen donor had a strong main effect on pollen-tube growth rate when both donor and recipient were considered to be fixed effects ($P < 0.01$ for donor 37 versus 41, and 51 versus 53; $P < 0.001$ for 45 versus 63 (both years), 54 versus 60, and 53 versus 60). When donors and recipients were considered to be random effects (interaction mean square as the *F*-ratio denominator), donor was still significant ($P < 0.01$) for all pairs except 51 versus 53. In both types of analysis, the interaction between donor and recipient was never significant. Recipient was often significant as a main effect, but this is not relevant to competition between donors within styles. Consistent donor effects across recipients are also documented in Fig. 2. ID, identification number of each plant.

mendelian genetic marker to show that differences in pollen-tube growth rate lead to nonrandom fertilization, as expected⁹. Therefore, we conclude that male fecundity can indeed be influenced by pollen competitive ability when mixed pollen loads occur on stigmas.

This study provides the strongest evidence to date for effects of pollen-tube competition on the number of seeds sired by plants in natural populations. The phenomenon could be widespread, as variation in pollen-tube growth rates has also been

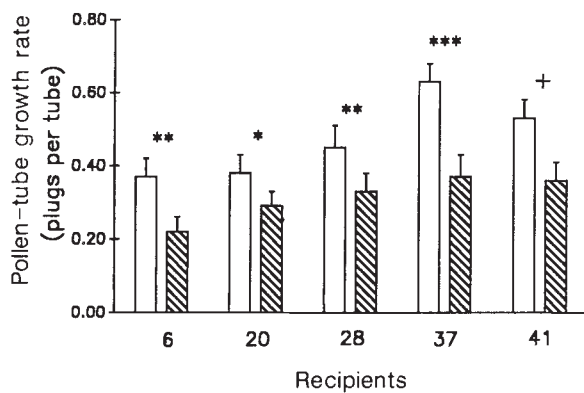
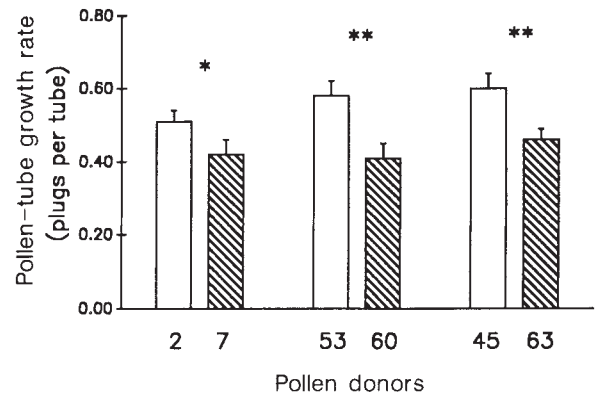


FIG. 1 Comparisons of relative pollen-tube growth rate for two pollen donors crossed with five recipient plants. Faster-growing pollen-tubes had more callose plugs (after 3 h). Mean ± 1 s.e.; sample sizes, paired *t*-tests, and significance levels as given in Table 1. Hatched bars, donor 60; open bars, donor 54.

FIG. 2 Comparisons of relative pollen-tube growth rate for three pairs of pollen donors. Each pair was tested on one flower of 14–15 unrelated recipients. Mean ± 1 s.e.; statistical tests as in Fig. 1; open bars designate donor with faster-growing tubes.



seen in other species^{10,11}. But most reports of nonrandom fertilization refer only to comparisons between self and outcross pollen^{12–14} or comparisons among pollen sources from different populations^{15,16}. A few previous studies demonstrated non-random fertilization following outcross hand-pollination^{17–19}, but the mechanism for this process was not identified. The evolutionary role of pollen-tube competition is often discussed in the literature, yet we know almost nothing about whether it occurs in nature.

To our knowledge, this study is the first to demonstrate that differences in pollen-tube growth rates are consistent across maternal genotypes. Sexual selection is therefore possible because 'super-males' with faster-growing pollen-tubes could potentially sire a disproportionately large number of seeds on a wide range of maternal plants. For simplicity, we describe this potential form of sexual selection as being due to pollen–pollen competition, but we do not mean to imply that pollen–style interactions and 'female choice' are unimportant. Discriminating between male–male competition and female choice is particularly difficult in plants, especially with regard to processes that take place after pollen arrives at stigmas^{3,20–22}.

To fully understand the evolutionary implications of our findings, we need to know the extent to which ecological factors

such as pollinator behaviour influence male reproductive success^{3,23}. We must also ascertain whether pollen-tube growth rate is heritable and, if so, what maintains heritable variation in this fitness-related trait. Despite these gaps in our knowledge, documenting that individuals vary in pollen competitive ability is an important first step toward identifying mechanisms for nonrandom mating.

In a widely cited paper, Mulcahy proposed that pollen-tube competition played a creative part in the rapid evolution of angiosperms²⁴. He noted that the angiosperm style provides a selective arena in which deleterious mutations might retard pollen-tube growth and thereby be eliminated from the gene pool. Furthermore, individuals with faster-growing pollen may produce faster-growing progeny because of overlapping gene expression in pollen and sporophytes^{24,25}. In existing populations, however, effects of pollen-tube competition on offspring vigour seem to be relatively small^{26,27}. Our results point to the intriguing possibility that pollen-tube competition has a more important effect on the number of offspring that a plant can sire. We hypothesize that when pollinators deliver surplus pollen to flowers, plant fitness is influenced by relative pollen-tube growth rates. □

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Is colour vision possible with only rods and blue-sensitive cones?

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AT night all cats are grey, but with the approach of dawn they take on colour. By starlight, a single class of photoreceptors, the rods, function, whereas by daylight, three classes, the blue-, green- and red-sensitive cones, are active and provide colour vision. Only by comparing the rates of quantal absorption in more than one photoreceptor class is colour vision possible. Although the comparisons generally take place between the cones, they can involve the rods as well¹⁻⁴. Here we investigate the wavelength discrimination of an extremely rare group of individuals, blue-cone monochromats, who have only rods and one class of cones⁵⁻⁸. We find that these individuals can distinguish wavelengths (440 to 500 nm) in the twilight region where the rods and blue-sensitive cones are simultaneously active.

To determine whether colour vision is possible with only rods and a single class of cones, we chose as observers five males with the rare genetic disorder known as blue-cone monochromacy or X-chromosome-linked incomplete achromatopsia⁵⁻⁹. The vision of all five of the observers can be fully described by the participation of normally functioning rods under scotopic conditions and normally functioning blue-sensitive cones under photopic ones^{5,7,8}. Peripheral green and/or red cone function, which may be residual in many of the affected males in pedigrees of blue-cone monochromacy¹⁰, was excluded by colour matching, and by spectral sensitivity^{7,8} and transient tritanopia^{7,11,12} measurements, made under chromatic adaptation conditions.

The psychophysical absence of the green- and red-cone sensitivities accords with the molecular genetics. Individual DNA blot hybridization patterns (J. Nathans, personal communication) reveal that the five observers have alternations in the red and green visual pigment gene cluster of their X chromosomes. The pattern in four of the observers, F.B., S.B., M.P. and K.S., corresponds to the more common of two recently reported⁹, in which only one of the tandem array of red and green visual pigment genes remains and is rendered nonfunctional by a point mutation. In the fifth observer, P.S., the pattern is of the second type: both the red and green visual pigment genes are nonfunctional owing to the deletion of DNA.

Despite the loss of the green- and red-cone spectral sensitivities, all five blue-cone monochromats claim to have some colour vision, but as we have already shown⁸, their self reports are unreliable. Further, their apparently normal performances on some portions of traditional colour discrimination tests at photopic levels can be explained by luminosity judgements mediated by their blue-sensitive cones⁸. The question of colour vision can be settled, however, by wavelength discrimination experiments, in which the observer is required to find the minimal or just noticeable difference (JND) in wavelength that allows him to distinguish two spectral stimuli.

As in typical wavelength discrimination experiments¹³, our five blue-cone monochromat observers viewed a bipartite field, one half of which was filled with light of a standard wavelength, the other with light of a comparison wavelength (see inset to Fig. 1c). To ensure that the observers could not discriminate the half-fields on the basis of brightness, the luminances of the half-fields were equated according to each observer's spectral sensitivity function measured beforehand by heterochromatic brightness matching under the same stimulus conditions¹⁴.

The results for an adapting standard of 8 trolands (td) are given in Fig. 1. Panel *a* shows the average heterochromatic brightness matching functions obtained for the five blue-cone monochromats and for 8 normal trichromats. The normal observers' function has a broad peak near 550 nm, corresponding to the maximum of the Commission Internationale d'Eclairage (CIE, 1978) average luminous efficiencies obtained by direct heterochromatic brightness matching (dotted line)¹⁵ (which is dominated by the green- and red-sensitive cones). That of the blue-cone monochromats has a peak near 520 nm, corresponding to the maximum of the CIE (1951) scotopic luminosity function (large dashed line)¹⁵. At long wavelengths the two curves diverge in accordance with the photopic and scotopic functions. But, at short wavelengths, both are more sensitive than either the photopic or scotopic functions. The cause of this is revealed in Fig. 1b, which displays the individual brightness curves of the five blue-cone monochromats. At wavelengths shorter than 500 nm, one of the curves (F.B.) deviates strikingly from the function, indicating the emergence near 450 nm of a second peak, which is well fitted by the blue- (or short-wavelength-sensitive) cone spectral sensitivity function of Smith and Pokorny¹⁶ (short dashed line). Thus, at this intensity level, some blue-cone monochromats are primarily using rods to generate their brightness matching function (even though their blue-cones are also active), whereas others are using both the blue-cones and rods to do so.

Figure 1c shows the average wavelength discrimination functions of the blue-cone monochromat and normal observers obtained at the same intensity level (8 td). JNDs in wavelength are plotted as a function of the standard wavelength. The average normal curve has the same general appearance as reported classic curves¹³: a relative maximum is observed at about 530 nm, two relative minima at approximately 490 and 590 nm. The average curve for the blue-cone monochromats, on the other hand, has only one minimum between 460 and 480 nm, and wavelength discrimination completely collapses for wavelengths longer than 520 nm. Within the narrow range between 440 and 500 nm, however, their discriminability compares favourably with that of the normal observers. The individual wavelength discrimination functions of the five blue-cone monochromat observers are shown in Fig. 1d.

Figure 2 shows the heterochromatic brightness matches and wavelength discrimination functions of observer P.S. at three mesopic adaptation levels below rod saturation: 0.8, 8 and 80 td. Clearly, wavelength discrimination extends to longer wavelengths as the field intensity is increased, but the best performance (that is, the smallest JND) is obtained at the intermediate intensity of 8 td. The change can be related to his spectral sensitivity functions. As the rod peak declines in magnitude relative to the blue-sensitive cone peak, the sensitivities of the two receptor types first converge then diverge and the spectral region where the sensitivities are most similar shifts from the short to the middle wavelengths. At higher light levels (>800 td), where the rods saturate, his spectral luminosity function (and those of the other blue-cone monochromats) becomes unimodal, with a peak near 450 nm, and, wavelength discrimination accordingly breaks down.

Thus, the answer to the question posed by the title is yes, within a limited intensity range, colour vision is possible with only rods and blue-sensitive cones. This finding is interesting for two reasons. First, previous demonstrations of rudimentary colour vision in alleged blue-cone monochromats^{17,18} have relied on the assumption that they have a third receptor type—a rhodopsin cone, which is used in conjunction with the blue-sensitive cones to discriminate colours. The presence of such receptors has not been verified psychophysically in the five blue-cone monochromats investigated here^{7,8}. This accords with the original explanation of blue-cone monochromacy⁵, and with the molecular genetics⁹. Second, it has been proposed that rod signals travel in pathways carrying signals from the blue-sensi-

tive cones^{19,20}, largely because rod vision at dusk and dawn takes on a bluish tinge. If a common neural pathway exists then it suggests that colour vision with only rods and blue-sensitive cones should not be possible; because the two types of signals are being summed rather than compared. We find the contrary, at least in the blue-cone monochromat. This implies that some rod and cone signals travel by separate pathways to the visual processing stage where wavelength discrimination takes place.

We conclude that vestigial colour vision is present in blue-cone monochromats, at intensity levels where both their rods and blue-cones are simultaneously active. This raises the issue of

whether the rods generally interact with cones to contribute to colour vision. Previously, it has been demonstrated that at mesopic levels, when the field of view is enlarged to include the parafoveal retina, human colour vision is tetrachromatic: to maintain colour matches at more than one light level, four independent variables are required instead of the usual three^{2,4,21}. (At any one level, colour vision is never more than trichromatic.) Further, it has been demonstrated that both protanopic and deuteranopic dichromats become trichromatic under similar conditions²². A plausible interpretation of these findings as well as our own is that rod signals travel along the

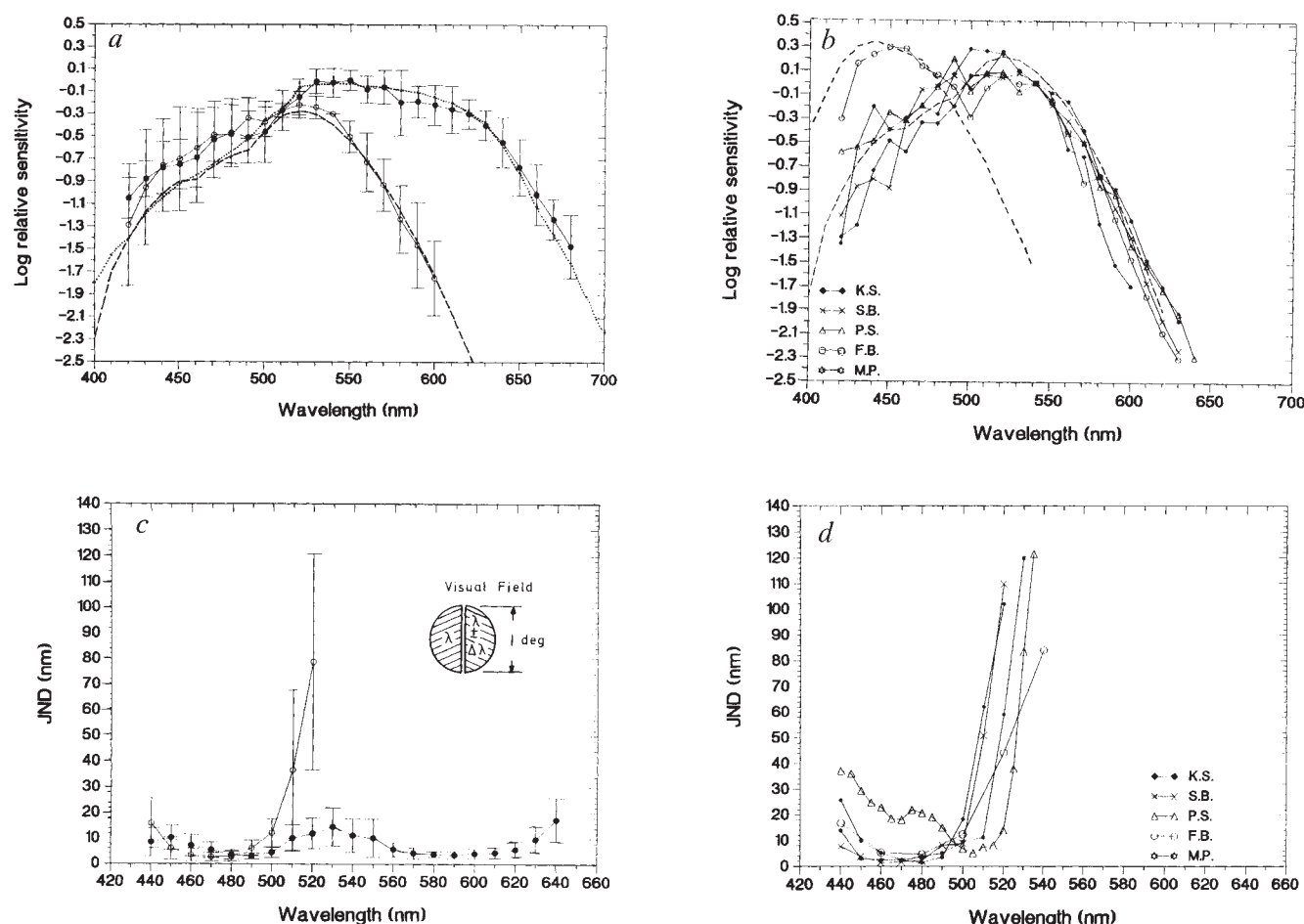


FIG. 1 *a*, The average brightness matches of 5 blue-cone monochromats (age 9–53; open circles) and of 8 normal trichromats (age 21–32; filled circles). The data points are shown with a bar corresponding to one standard deviation. The matches were made in a bipartite photometric field (see inset, Fig. 1*c*) presented in Maxwellian view. The observer was required to find by a staircase tracking procedure the luminance of the comparison half-field (as a function of wavelength) needed to match the brightness of a 540 nm standard half-field set to a retinal illuminance of 8 td. Both halves of the field, which subtended 1° of visual angle in diameter, were presented simultaneously for 1 s. The standard and comparison wavelengths were selected by means of grating monochromators (Jobin Yvon H.10 Vis), with a full-width at half-amplitude of less than 4 nm (for other details, see ref. 14). The dotted line indicates the quantized CIE (1978) average luminous efficiencies obtained by direct heterochromatic brightness matching¹⁵; the large-dashed line indicates the CIE (1951) scotopic luminosity function, quantized and corrected for a modest amount of macular pigment absorption¹⁵. The correction to the CIE scotopic function is required since it pertains to the peripheral retina, which is not screened by the short-wave absorbing macular pigment, whereas the blue-cone monochromat brightness matches pertain to the central fovea, which is. The correction for macular absorption was made according to standard mean optical density values¹⁵, assuming a maximum density of 0.5 log₁₀ unit at 458 nm. *b*, The individual heterochromatic

brightness matches of the 5 blue-cone monochromats. Each curve has been separately normalized to the sensitivity value obtained at the standard wavelength (540 nm). The small-dashed line indicates the Smith and Pokorny short-wave (blue) cone sensitivity function¹⁶. *c*, The average wavelength discrimination curves of the five blue-cone monochromats (open circles) and of the eight normal trichromats (filled circles) measured at a retinal illuminance of 8 td. (JND, Just noticeable difference.) The inset shows the field of view, in which λ and $\lambda + \Delta\lambda$ indicate, respectively, the standard and comparison wavelengths. During an experiment, the wavelength of the comparison half-field was changed in 1-nm steps from equivalence with that of the standard half-field. The luminances of the two fields were held constant by reference to the observer's individual heterochromatic brightness functions (for details, see ref. 14). The discrimination threshold or JND in wavelength was found where the observer signalled that the two halves of the field were no longer identical in appearance. This procedure was repeated five times for a series of standard wavelength throughout the visible spectrum. Between presentations, the observer adapted for 3 s to a homogeneous white field of the same intensity as the standard and comparison half-fields. The luminance of the white adapting field was matched to that of the standard and comparison fields by means of heterochromatic brightness matching. *d*, The individual wavelength discrimination curves of the 5 blue-cone monochromats.

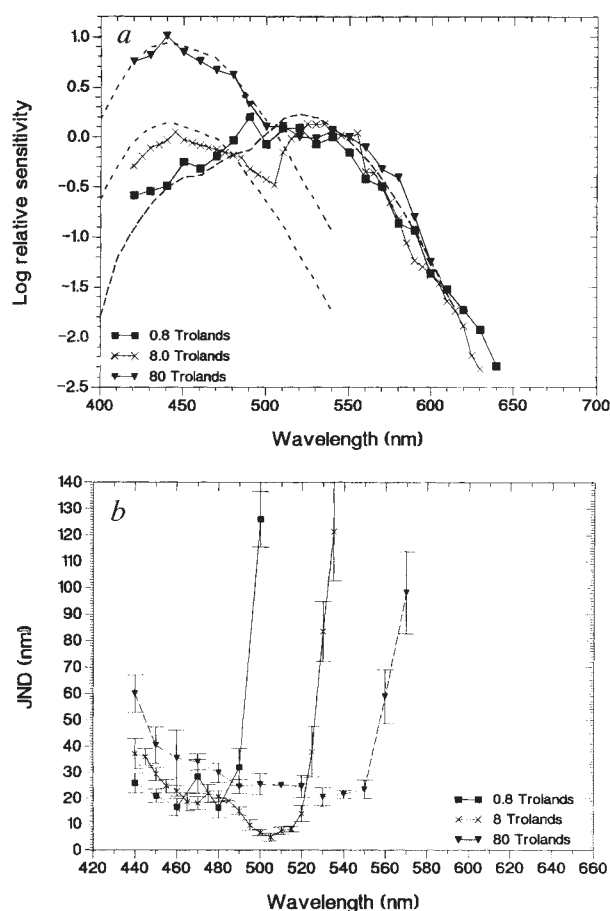


FIG. 2 *a*, The heterochromatic brightness matches of blue-cone monochromat P.S. measured at three retinal illuminances of the standard 540 nm field: 0.8, 8 and 80 td. The large-dashed line indicates the CIE (1951) scotopic luminosity function, quantized and corrected for macular pigment absorption; the small-dashed line, the Smith and Pokorny short-wave (blue) cone sensitivity function¹⁶. *b*, The wavelength discrimination functions of blue-cone monochromat P.S. measured at the same three levels as in *a*. The error bars indicate one standard deviation of the mean.

cone chromatically-opponent^{22–24} and non-opponent²⁵ pathways and do not have access to an independent channel^{26–29}. In normal trichromats, the input from the rods may only slightly alter the ongoing activity of these pathways, leading to tetrachromatic colour matching but causing no new or independent sensations. In dichromats and blue-cone monochromats, on the other hand, given the reduced number of their independent cone signals, such variable rod input may significantly modulate activity in the opponent pathways, permitting wavelength discrimination and leading to sensations that qualitatively differ from those attributable to their cones alone²². □

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Repeat I of the dihydropyridine receptor is critical in determining calcium channel activation kinetics

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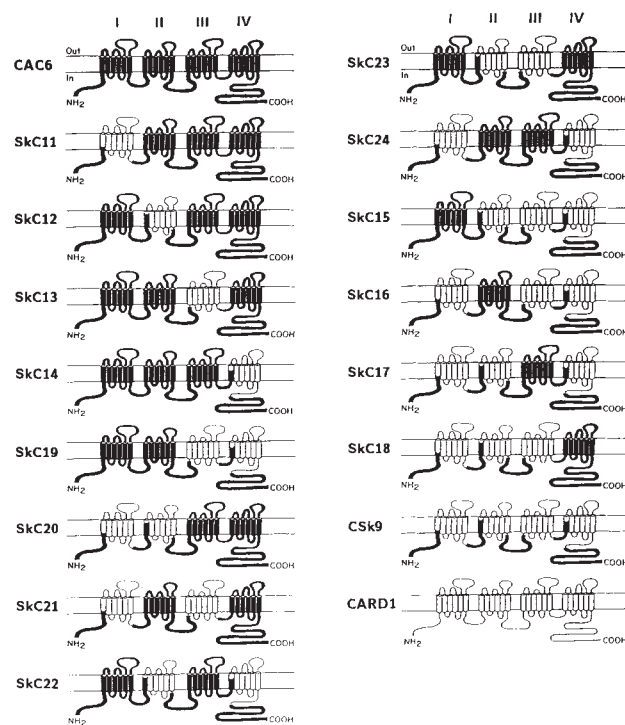
MEMBRANE depolarization causes many kinds of ion channels to open, a process termed activation¹. For both Na⁺ channels^{2–4} and Ca²⁺ channels^{5,6}, kinetic analysis of current has suggested that during activation the channel undergoes several conformational changes before reaching the open state. Structurally, these channels share a common motif⁷: the central element is a large polypeptide with four repeating units of homology (repeats I–IV), each containing a voltage-sensing region, the S4 segment^{8–11}. This suggests that the distinct conformational transitions inferred from kinetic analysis may be equated with conformational changes of the individual structural repeats⁸. To investigate the molecular basis of channel activation, we constructed complementary DNAs encoding chimaeric Ca²⁺ channels in which one or more of the four repeats of the skeletal muscle dihydropyridine receptor are replaced by the corresponding repeats derived from the cardiac dihydropyridine receptor. We report here that repeat I determines whether the chimaeric Ca²⁺ channel shows slow (skeletal muscle-like)¹² or rapid (cardiac-like)¹³ activation.

Expression of cDNAs encoding chimaeras with one or more of the large, putative cytoplasmic regions of the cardiac dihydropyridine (DHP) receptor replaced by corresponding regions of the skeletal muscle DHP receptor, showed that the putative cytoplasmic region linking repeat II and repeat III is a major determinant of skeletal muscle-type excitation-contraction coupling, whereas the four repeating units of homology, constituting the putative transmembrane and adjacent regions, are important in determining channel properties¹⁴. To examine the functional significance of each repeat in Ca²⁺ channel activation kinetics, we constructed 15 different expression plasmids carrying chimaeric DHP receptor cDNAs (Fig. 1). These expression plasmids were injected into skeletal muscle myotubes prepared from mice with the muscular dysgenesis mutation¹⁵. Myotubes expressing each of the chimaeric plasmids were identified on the basis of contraction in response to electrical stimulation¹²; all the chimaeric plasmids were functionally expressed, except pSkC21. The whole-cell variant of the patch-clamp technique revealed that all cells displaying contractile responses also expressed Ca²⁺ currents, although the level of expressed current differed for the different chimaeras. Specifically, the maximal

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FIG. 1 Schematic representation of the structures of chimaeric DHP receptors composed of sequences derived from the skeletal muscle DHP receptor (CAC6) and the cardiac DHP receptor (CARD1). For each DHP receptor, the four units of homology (repeats I–IV from left to right) are displayed linearly and the six putative transmembrane segments (S1–S6 from left to right) in each repeat are shown by cylinders. Portions of the constructs that are of skeletal muscle origin are indicated by filled cylinders and heavy lines, and portions of cardiac origin and junctional sequences common to the two parental DHP receptors are indicated by open cylinders and thin lines. The compositions of the individual chimaeric DHP receptors are as follows (Sk and C, skeletal muscle and cardiac DHP receptor, respectively; numbers in parentheses, amino-acid numbers^{10,17}; the junctional sequences common to the two DHP receptors are represented by amino-acid numbers of the cardiac DHP receptor). SkC11: Sk(1–55), C(159–464) and Sk(364–1,873). SkC12: Sk(1–448), C(571–787) and Sk(666–1,873). SkC13: Sk(1–791), C(923–1,204) and Sk(1,074–1,873). SkC14: Sk(1–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC19: Sk(1–791), C(923–1,204), Sk(1,074–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC20: Sk(1–55), C(159–464), Sk(364–448), C(571–787) and Sk(666–1,873). SkC21: Sk(1–55), C(159–464), Sk(364–791), C(923–1,204) and Sk(1,074–1,873). SkC22: Sk(1–448), C(571–787), Sk(666–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC23: Sk(1–448), C(571–787), Sk(666–791), C(923–1,204) and Sk(1,074–1,873). SkC24: Sk(1–55), C(159–464), Sk(364–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC15: Sk(1–448), C(571–787), Sk(666–791), C(923–1,204), Sk(1,074–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC16: Sk(1–55), C(159–464), Sk(364–791), C(923–1,204), Sk(1,074–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC17: Sk(1–55), C(159–464), Sk(364–448), C(571–787), Sk(666–1,129), C(1,261–1,634) and Sk(1,510–1,873). SkC18: Sk(1–55), C(159–464), Sk(364–448), C(571–787), Sk(666–791), C(923–1,204) and Sk(1,074–1,873). CSK9: Sk(1–55), C(159–464), Sk(364–448), C(571–787), Sk(666–791), C(923–1,204), Sk(1,074–1,129), C(1,261–1,634) and Sk(1,510–1,873).

METHODS. The expression plasmids pCAC6 (ref. 12) and pCARD1 (ref. 17), carrying the cDNAs encoding the skeletal muscle and the cardiac DHP receptor, respectively, have been described previously. The expression plasmids pSkC11–pSkC24 and pCSK9, carrying the cDNAs encoding the individual chimaeric DHP receptors, were constructed by inserting the corresponding cDNAs into the *HindIII* site of the plasmid pKCRH2 (ref. 25). The chimaeric DHP receptor cDNAs are composed of the following restriction fragments (the origin of the fragments is given in parentheses). pCSK9: 3.2-kilobase-pair (kb) *HindIII*–*BspHI* (pCSK7; see ref. 14), 0.24-kb *BspHI*–*BstXI* (pCAC6) and 2.3-kb *BstXI*–*HindIII* (pCSK7). pSkC11: 1.1-kb *HindIII*–*PvuII* (pCSK9) and 4.6-kb *PvuII*–*HindIII* (pCAC6). pSkC12: 1.1-kb *HindIII*–*PvuII* (pCAC6), 1.1-kb *PvuII*–*BstBI* (pCSK9) and 3.5-kb *BstBI*–*HindIII* (pCAC6). pSkC13: 2.2-kb *HindIII*–*BstBI*



(pCAC6), 0.99-kb *BstBI*–*BspHI* (pCSK9) and 2.5-kb *BspHI*–*HindIII* (pCAC6). pSkC14: 3.2-kb *HindIII*–*BspHI* (pCAC6) and 2.5-kb *BspHI*–*HindIII* (pCSK9). pSkC15: 2.2-kb *HindIII*–*BstBI* (pSkC12) and 3.5-kb *BstBI*–*HindIII* (pCSK9). pSkC16: 2.2-kb *HindIII*–*BstBI* (pSkC11) and 3.5-kb *BstBI*–*HindIII* (pCSK9). pSkC17: 2.2-kb *HindIII*–*BstBI* (pCSK9) and 3.5-kb *BstBI*–*HindIII* (pSkC14). pSkC18: 2.2-kb *HindIII*–*BstBI* (pCSK9) and 3.5-kb *BstBI*–*HindIII* (pSkC13). pSkC19: 2.2-kb *HindIII*–*BstBI* (pCAC6) and 3.5-kb *BstBI*–*HindIII* (pCSK9). pSkC20: 2.2-kb *HindIII*–*BstBI* (pCSK9) and 3.5-kb *BstBI*–*HindIII* (pCAC6). pSkC21: 2.2-kb *HindIII*–*BstBI* (pSkC11) and 3.5-kb *BstBI*–*HindIII* (pSkC13). pSkC22: 2.2-kb *HindIII*–*BstBI* (pSkC12) and 3.5-kb *BstBI*–*HindIII* (pSkC14). pSkC23: 2.2-kb *HindIII*–*BstBI* (pSkC12) and 3.5-kb *BstBI*–*HindIII* (pSkC13). pSkC24: 2.2-kb *HindIII*–*BstBI* (pSkC11) and 3.5-kb *BstBI*–*HindIII* (pSkC14).

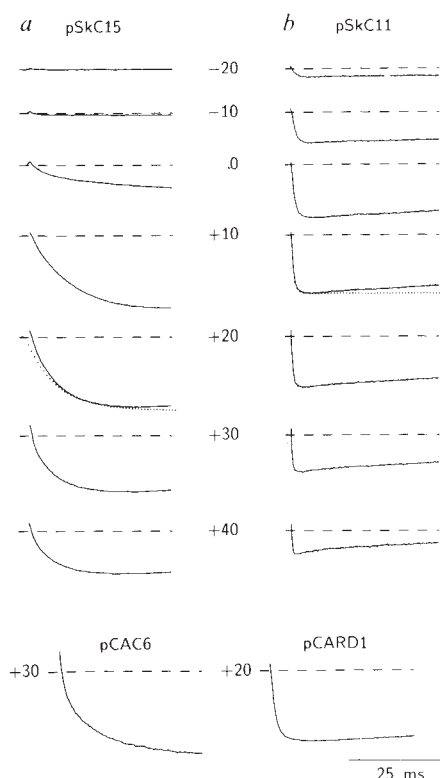


FIG. 2 Ca^{2+} currents expressed in dysgenic myotubes injected with pSkC15 (a) and pSkC11 (b). The insets on the bottom left and right, respectively, illustrate Ca^{2+} currents recorded from a pCAC6-injected myotube and a pCARD1-injected myotube. Test potentials are indicated next to the current traces. The dotted curves represent the single exponential function

$$I(t) = I_{\infty} [1 - \exp(-t/\tau_{\text{act}})], \quad (1)$$

where $I(t)$ is the current at time t after stepping membrane potential from the holding potential to V_{test} , I_{∞} is the steady-state current and τ_{act} is the time constant of activation. The exponential curves were calculated with $\tau_{\text{act}} = 20$ ms (pSkC15, $V_{\text{test}} = +20$ mV) and 2.5 ms (pSkC11, $V_{\text{test}} = +10$ mV). The initial and final portions of the current traces deviate from the fitted exponentials due at least partly to the presence of intramembrane charge movement²⁶ and inactivation, respectively. Vertical calibration corresponds to 10 nA (pSkC15), 2.5 nA (pSkC11), 3 nA (pCAC6) or 10 nA (pCARD1). a, Cell CD03, linear capacitance (C) = 685 pF; b, cell CC67, (C) = 415 pF; pCAC6, cell CC57, (C) = 1,000 pF; pCARD1, cell CE11, (C) = 645 pF.

METHODS. The procedures for whole-cell voltage clamp²⁷ measurement of Ca^{2+} currents were as previously described^{12,13}. The patch pipette contained (in mM): 140 Cs-aspartate, 5 MgCl_2 , 10 Cs_2 -EGTA, 10 HEPES (pH 7.4 with CsOH). The bath contained (in mM): 10 Ca^{2+} , 145 tetraethylammonium ion, 165 Cl^- , 0.003 tetrodotoxin, 10 HEPES (pH 7.4 with CsOH). Before the application of any specialized pulse protocol to a cell, Ca^{2+} currents were first measured with the standard protocol, a step directly from the holding potential (-80 mV) to the test potential (V_{test}), for $V_{\text{test}} = -30$ to $+60$ mV, in 10 mV increments. The test pulse was preceded by a 1 s prepulse to -30 mV to inactivate T-type Ca^{2+} current²⁶ in those cells where it was appreciable in size. All experiments were carried out at room temperature (20 – 22°C).

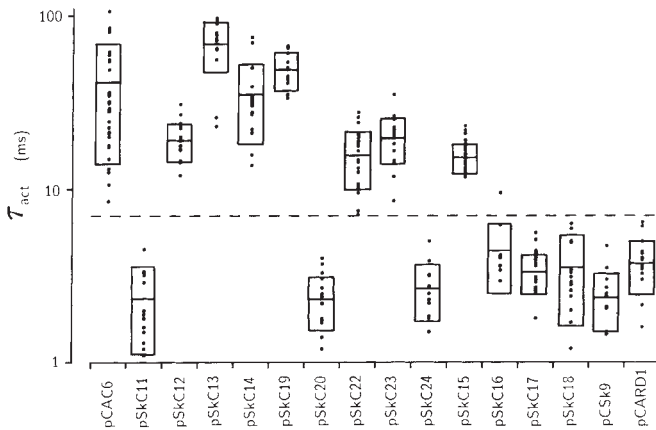


FIG. 3 Time constant of activation (τ_{act}) of Ca^{2+} current expressed in dysgenic myotubes injected with expression plasmids carrying the skeletal muscle (pCAC6), cardiac (pCARD1) and chimaeric (pSkC11–pSkC20, pSkC22–pSkC24 and pCSK9) DHP receptor cDNAs. Individual data points are plotted as filled circles (overlapping values are plotted as single points), the short horizontal line indicates the mean, and the box indicates $\pm$ s.d. Note that values of τ_{act} for all the constructs with repeat I of skeletal muscle origin were greater than 7 ms (indicated by dashed horizontal line), and values of τ_{act} for all the constructs with repeat I of cardiac origin were <7 ms (with the exception of one data point). Currents were evoked with the standard protocol with V_{test} ranging from -30 to $+60$ mV, in 10 mV increments. Values of τ_{act} were determined by fitting the activation phase of each current according to equation (1). Values of τ_{act} plotted here are for the V_{test} at or 10 mV positive to the potential that elicited maximal inward current in a given myotube. $n=34, 16, 18, 13, 17, 14, 15, 21, 18, 12, 32, 9, 25, 18, 11$ and 15 determinations, respectively, for constructs in the order indicated along the abscissa of the Figure.

inward current (normalized by linear capacitance) varied from a minimum of 2.6 ± 1.2 pA pF $^{-1}$ (mean $\pm$ s.d., $n=12$) for pSkC16 to a maximum of 14.9 ± 8.4 pA pF $^{-1}$ ($n=12$) for pSkC15.

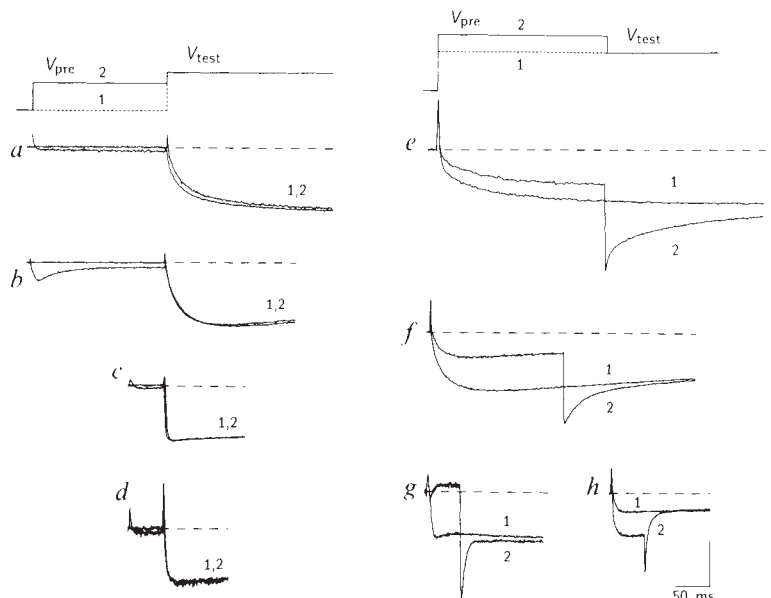
Figure 2 illustrates Ca^{2+} currents resulting from expression of pSkC15 (in which repeat I is skeletal and repeats II–IV are cardiac) and pSkC11 (in which repeat I is cardiac and repeats II–IV are skeletal). The current produced by pSkC15 (Fig. 2a) activates slowly, resembling the current produced by pCAC6, the entirely skeletal muscle DHP receptor (Fig. 2, bottom left). By contrast, the current produced by pSkC11 (Fig. 2b) activates rapidly, resembling that for pCARD1, the entirely cardiac DHP receptor (Fig. 2, bottom right). The measured currents could be approximated by a single exponential function (for details, see legend to Fig. 2), providing a simple measure (τ_{act}) of the activation kinetics for different constructs. Because the rate of activation varies with test potential, τ_{act} was determined in each myotube expressing a cDNA for a test potential (V_{test}) at or just above the potential that elicited maximal inward current. Figure 3 illustrates τ_{act} for the chimaeric constructs as well as for pCAC6 and pCARD1. For any given plasmid, τ_{act} shows a considerable range of values. But the data clearly fall into two groups with almost no overlap: τ_{act} for pCAC6, pSkC12–15, pSkC19, pSkC22 and pSkC23 is large (that is, activation is slow) compared with that for pCARD1, pSkC11, pSkC16–18, pSkC20, pSkC24 and pCSK9. The only consistent, structural feature that distinguishes these two groups of chimaeric plasmids is repeat I (see Fig. 1). For all chimaeric plasmids in which repeat I is of skeletal muscle

origin, τ_{act} is large, whereas for those in which repeat I is of cardiac origin, τ_{act} is small.

The slow time course of current when repeat I is skeletal indicates that one or more slow transitions must occur during activation. If several slow transitions precede opening, a near-threshold prepulse might be expected to speed the rate of activation of current produced by a subsequent, stronger test depolarization. This was not the case for pCAC6 and pSkC15: the current elicited when V_{test} was applied directly from the holding potential (-80 mV), and the current elicited when this same V_{test} was applied after a near-threshold prepulse, had almost identical time courses (Fig. 4a, b). For pSkC11 and pCARD1, activation kinetics were also little affected by a near-threshold prepulse (Fig. 4c, d). Thus, if voltage-dependent transitions occur between -80 mV and the prepulse potential, they must occur rapidly.

Figure 4e–h illustrates 'tail currents' elicited when voltage was first stepped to a strongly depolarized prepulse potential (V_{pre}) and then back to V_{test} . Because the steady-state probability of channels being open is greater at V_{pre} , the current after stepping back to V_{test} is initially increased in size. This increased current decays to the equilibrium value with a time course comparable to that for activation at the same V_{test} . There was similar agreement between activation and tail current kinetics over the limited range of potentials where both could be determined. Thus, when only repeat I is skeletal (pSkC15), the kinetics of both activation and tail current decay are slow, just

FIG. 4 Ca^{2+} currents expressed in dysgenic myotubes injected with pCAC6 (a, e), pSkC15 (b, f), pSkC11 (c, g) and pCARD1 (d, h), measured with voltage protocols diagrammed at top. a–d, currents were measured either with the standard protocol (traces labelled 1) or with the prepulse protocol in which V_{test} was preceded by a prepulse (V_{pre}) to a near-threshold potential (traces labelled 2). e–h, Currents were measured either with the standard protocol (traces labelled 1) or with the tail current protocol in which V_{test} was preceded by a prepulse (V_{pre}) to a strongly depolarized potential (traces labelled 2). Note that the durations of the prepulses and test pulses varied for the different constructs (see below). Vertical calibration 1.5, 4.0, 2.0, 0.5, 1.0, 4.0, 1.0 and 2.0 nA, respectively, in a (cell BL20, $C=445$ pF, $V_{pre}=0$ mV for 200 ms, $V_{test}=+40$ mV), b (cell BK69, $C=420$ pF, $V_{pre}=-10$ mV for 200 ms, $V_{test}=+10$ mV), c (cell BL05, $C=460$ pF, $V_{pre}=-25$ mV for 50 ms, $V_{test}=+10$ mV), d (cell BK84, $C=105$ pF, $V_{pre}=-20$ mV for 50 ms, $V_{test}=+10$ mV), e (cell BK76, $C=360$ pF, $V_{pre}=+70$ mV for 250 ms, $V_{test}=+30$ mV), f (cell BK69, $C=420$ pF, $V_{pre}=+60$ mV for 200 ms, $V_{test}=+10$ mV), g (cell BL05, $C=460$ pF, $V_{pre}=+80$ mV for 50 ms, $V_{test}=-5$ mV) and h (cell BK84, $C=105$ pF, $V_{pre}=+50$ mV for 50 ms, $V_{test}=+10$ mV).



as they are for the entirely skeletal muscle DHP receptor (pCAC6). Conversely, when only repeat I is cardiac (pSkC11), both activation and tail current kinetics are rapid, just as they are for the entirely cardiac DHP receptor (pCARD1).

We have shown here that repeat I of the DHP receptor has a critical role in governing Ca^{2+} channel activation kinetics. Several physical mechanisms might account for our kinetic results. One simple model is to suppose that each of the four repeats can independently and reversibly interconvert between testing and activated configurations, that depolarization favours the activated configuration and that all four repeats must be in the activated configuration for the channel to open. This model is similar to that originally proposed by Hodgkin and Huxley for the activation of Na^+ and K^+ channels in the squid giant axon² and is similar to the model recently proposed for the activation of A-type K^+ channels in *Drosophila* muscle¹⁶. With the model of four independent repeats, one can explain the kinetic behaviours of the chimaeric Ca^{2+} channels by assuming that the resting-to-activated interconversion of repeat I is slow when it is skeletal and rapid when it is cardiac. Alternatively, the identity of repeat I might affect the resting-to-activated interconversion of other repeats.

The amino-acid sequences of repeat I of the skeletal muscle¹⁰ and cardiac¹⁷ DHP receptors are markedly similar but there are several regions that differ, including the region between segments S5 and S6. Mutations in this region of a Na^+ channel¹⁸ and in the corresponding region of K^+ channels¹⁹⁻²³ strongly affect pharmacological sensitivities and ion permeation, which suggests that this portion of the protein is close to the channel pore or constitutes part of the channel lining (see also ref. 24). Other regions with differing amino-acid sequences are scattered

throughout repeat I. It remains to be determined which region of repeat I is responsible for the difference in activation kinetics between the skeletal muscle and the cardiac L-type Ca^{2+} channel. □

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Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A

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CYCLOSPORIN A and FK506 inhibit T- and B-cell activation and other processes essential to an effective immune response¹⁻³. In T lymphocytes these drugs disrupt an unknown step in the trans-mission of signals from the T-cell antigen receptor to cytokine genes that coordinate the immune response⁴⁻⁶. The putative intracellular receptors for FK506 and cyclosporin are *cis-trans* prolyl isomerases⁷⁻¹¹. Binding of the drug inhibits isomerase activity^{8,10,11}, but studies with other prolyl isomerase inhibitors¹² and analysis of cyclosporin-resistant mutants in yeast suggest that the effects of the drug result from the formation of an inhibitory complex between the drug and isomerase^{13,14}, and not from inhibition of isomerase activity. A transcription factor, NF-AT, which is essential for early T-cell gene activation, seems to be a specific target of cyclosporin A and FK506 action because transcription directed by this protein is blocked in T cells treated with these drugs, with little or no effect on other transcription factors such as AP-1 and NF- κ B (refs 15-17). Here we demonstrate that NF-AT is formed when a signal from the antigen receptor induces a pre-existing cytoplasmic subunit to translocate to the nucleus and combine with a newly synthesized nuclear subunit of NF-AT. FK506 and cyclosporin A block translocation of the cytoplasmic component without affecting synthesis of the nuclear subunit.

Using the human Jurkat T-cell line¹⁸, we have developed an

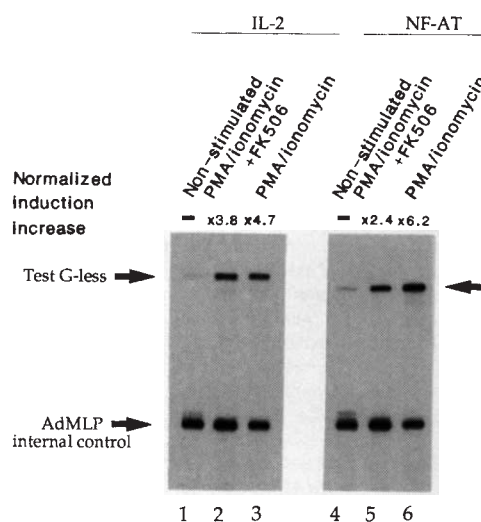


FIG. 1 *In vitro* transcription directed by the IL-2 enhancer or three tandemly linked NF-AT binding sites in nuclear extracts stimulated under different conditions. *a*, IL-2 promoter-directed transcription: lane 1, non-stimulated; lane 2, PMA/ionomycin/FK506-treated cells; and lane 3, PMA/ionomycin-stimulated cells; NF-AT directed transcription: lane 4, non-stimulated; lane 5, PMA/ionomycin/FK506-treated cells; lane 6, PMA/ionomycin-stimulated cells. Expression from the IL-2 enhancer and NF-AT G-less template generates 401- and 383-nucleotide transcripts, respectively (arrows). The adenovirus major late promoter (AdMLP) internal control generates a 280-nucleotide transcript. The induction increase is calculated after normalizing to AdMLP transcription.

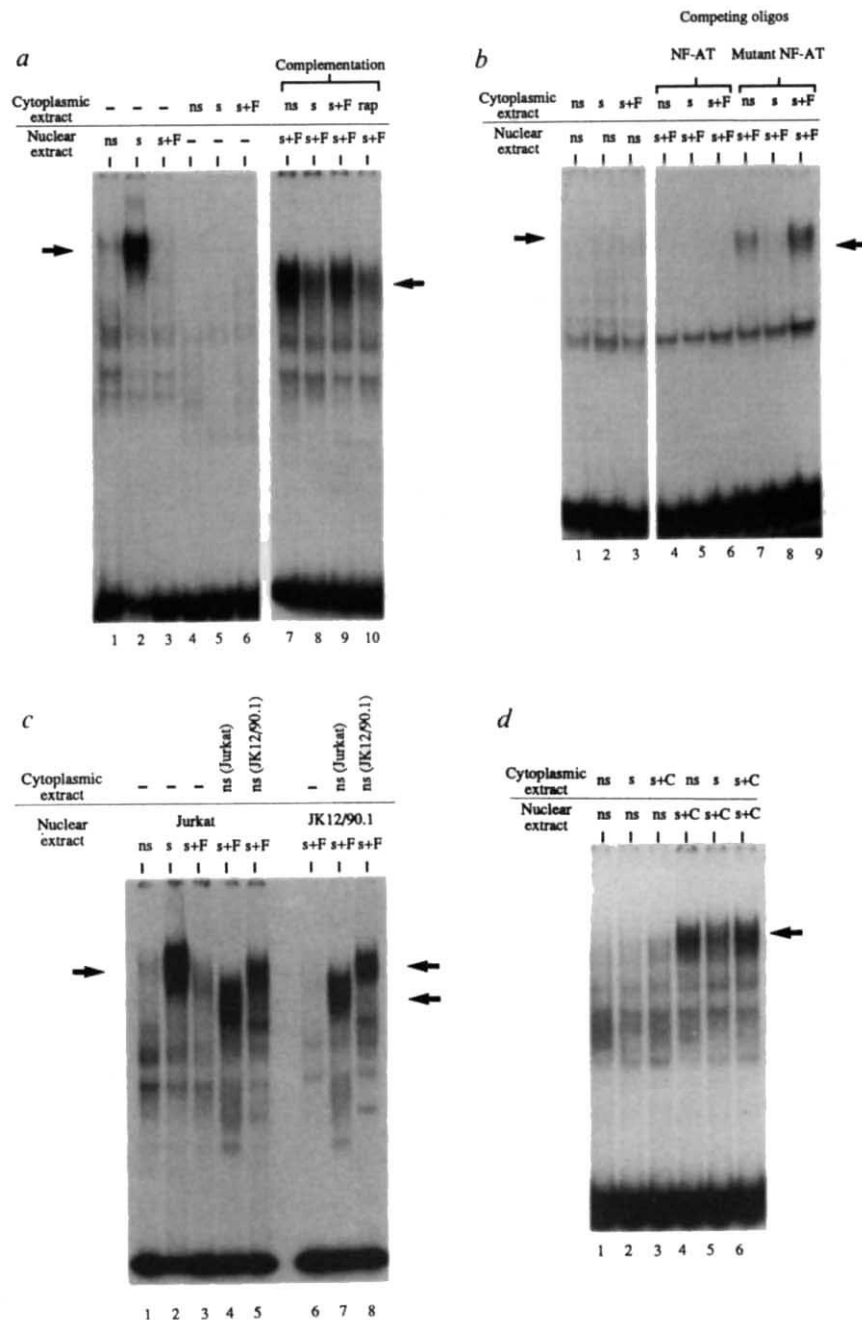
METHODS. Promoter constructs, nuclear extracts and transcription reactions were prepared as described (W.M.F. and G.R.C., manuscript submitted). NFAT Jurkat cells¹⁹ derived from a human T-cell leukaemia were stimulated with 20 ng ml⁻¹ PMA (Sigma), 2 μ M ionomycin (Calbiochem) for 2 h. FK506 (gift from S. Schreiber) was used at 10 ng ml⁻¹ and added 5 min before PMA and ionomycin. Transcription was quantitated using a radioanalytical imaging system (AMBIS).

activation-dependent, T-cell-specific *in vitro* transcription system that faithfully reflects the complex requirements for interleukin-2 (IL-2) transcription and T-cell activation (W.M.F. and G.R.C., manuscript submitted). Nuclear extracts from stimulated Jurkat cells transcribe an IL-2 enhancer template that lacks

guanine at levels fivefold higher than extracts from unstimulated cells (Fig. 1a). Unexpectedly, nuclear extracts from Jurkat cells that had been stimulated for 2 h with phorbol myristylacetate (PMA) and ionomycin in the presence of FK506 (10 ng ml⁻¹) transcribe the IL-2 G-less template in amounts nearly equivalent

FIG. 2 NF-AT binding activity can be quantitatively reconstituted from nuclear and cytoplasmic fractions of stimulated/FK506- and stimulated/cyclosporin A (CsA)-treated Jurkat cells. *a*, In lanes 1–6, nuclear extracts (10 µg) and cytoplasmic extracts (10 µg) from non-stimulated (ns), stimulated (s) with PMA/ionomycin, and stimulated/FK506-treated (s+F) cells were tested for NF-AT binding activity using electrophoretic gel mobility shift assays. In lanes 7–10, NF-AT was reconstituted by mixing nuclear and cytoplasmic extracts. Stimulated/FK506-treated (s+F) nuclear extracts (5 µg) were complemented with cytoplasmic extracts (5 µg) from: lane 7, non-stimulated (ns); lane 8, stimulated (s); lane 9, stimulated/FK506-treated (s+F); and lane 10, stimulated/rapamycin-treated (rap) cells. Arrows indicate the migration of the NF-AT DNA-protein complex in *a*, *b*, *c* and *d*. *b*, In lanes 1–3, mixing of nuclear extracts from nonstimulated cells (5 µg) with any cytoplasmic extracts (5 µg) fails to reconstitute NF-AT binding. In lanes 4–9, reconstituted NF-AT binding activity demonstrates DNA-binding specificity: nuclear extracts (5 µg) from stimulated/FK506-treated cells (s+F) were mixed with cytoplasmic extracts (5 µg) and competition was carried out with 10 ng of unlabelled NF-AT or mutant NF-AT oligonucleotide. *c*, The effect of FK506 was tested on a murine T-cell hybridoma, JK12/90.1 (ref. 23). In lanes 1–3, nuclear extracts (10 µg) from non-stimulated (ns), stimulated (s) with PMA/ionomycin, and stimulated/FK506-treated (s+F) Jurkat cells were tested for NF-AT binding activity. Lane 6 shows NF-AT binding in nuclear extracts from stimulated/FK506-treated (s+F) JK12/90.1 cells. In lanes 4–5 and 7–8, NF-AT is reconstituted by mixing nuclear extracts (5 µg) from stimulated/FK506-treated Jurkat or JK12/90.1 cells with cytoplasmic extracts (5 µg) from non-stimulated Jurkat or JK12/90.1 cells. *d*, In lanes 1–3, nuclear extracts (5 µg) from non-stimulated cells were mixed with cytoplasmic extracts (5 µg) from non-stimulated (ns), stimulated (s), and stimulated/CsA-treated (s+C) cells. Stimulated/CsA-treated (s+C) nuclear extracts (5 µg) were complemented with cytoplasmic fractions (5 µg) from: lane 4, non-stimulated (ns); lane 5, stimulated (s); and lane 6, stimulated/CsA-treated (s+C) cells.

METHODS. NFAT Jurkat cells and JK12/90.1 cells (a gift from N. Shastri) were stimulated for 2 h with 20 ng ml⁻¹ PMA and 2 µM ionomycin. To block NF-AT formation quantitatively, FK506 100 ng ml⁻¹ or CsA (Sandoz) 500 ng ml⁻¹ were used 5 min before addition of PMA and ionomycin without any toxic effects to the cells. Nuclear extracts were prepared as described³² with modifications¹⁹. Cytoplasmic extracts were made from the same cells as the nuclear extracts. Following lysis of the cells with buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF) plus 0.05% Nonidet P-40, and pelleting of the nuclei, the cytoplasmic fraction was removed and stabilized with 10% (v/v) glycerol and 1/10 volume of buffer B (0.3 M HEPES, pH 7.8, 1.4 M KCl and 30 mM MgCl₂). The cytoplasmic extract was centrifuged at 200,000g for 15 min. An equal volume of 3 M (NH₄)₂SO₄, pH 7.9, was added to the supernatant, and precipitated proteins were pelleted at 100,000g for 10 min. The pelleted cytoplasmic proteins were resuspended in buffer C (50 mM HEPES, pH 7.6, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% (v/v) glycerol) and desalted by passage over a P6DG column (BioRad). Protein concentrations were determined using a BioRad protein assay kit. To assess the completeness of the nuclear and cytoplasmic fractionation, we assayed for Oct-1 (a constitutive nuclear located DNA binding protein)



binding activity and β -galactosidase (cytoplasmic) activity which is present in our stably transfected NF-AT β -galactosidase Jurkat cell line¹⁹ following stimulation. We found no Oct-1 binding activity in the cytoplasmic fraction and β -galactosidase activity in the cytosol was 3.7-fold higher than in the nuclear fraction (data not shown). Electrophoretic mobility shift assays were essentially as described^{33,34}. Binding reactions were carried out as before¹⁹. The total protein used in each binding reaction was 10 µg. The end-labelled binding site for NF-AT was derived from the human IL-2 enhancer (-285 to -255 base pairs). The oligonucleotide sequence is 5'-GATCGGAGGAAAACT-GTTTCATACAGAAGGCGT-3'. The mutant NF-AT probe differs from the NF-AT oligonucleotide at four contact guanosine residues. The sequence is 5'-GATCAAGAAAGGAGTAAAAAaTtTTTaATACAGAA-3'. The lower-case letters indicate the mutated residues. Competition with 10 ng of unlabelled oligonucleotide represents a 100- to 200-fold molar excess over labelled probe.

to extracts from fully stimulated Jurkat cells (Fig. 1a; compare lanes 2 and 3), even though transcription of the endogenous IL-2 gene is fully inhibited under these conditions¹⁷. As most of the inhibitory effects of cyclosporin A and FK506 on IL-2 gene activation are due to inhibition of NF-AT function^{16,17}, we investigated transcription directed by this protein. *In vitro* transcription directed by multimerized binding sites for the NF-AT complex was reduced by only 2.5-fold in nuclear extracts of stimulated/FK506-treated cells (Fig. 1a; compare lanes 5 and 6), despite the fact that NF-AT-dependent transcription is totally blocked in the cells used to prepare the extracts^{13,17}. NF-AT DNA-binding activity is reduced by ~75% (refs 13, 17), which is far less than the inhibitory effects on IL-2 gene expression (usually >99%; ref. 17), but commensurate with the effects on NF-AT-dependent *in vitro* transcription. Thus, stimulated/FK506-treated cells seem to make reduced amounts of NF-AT which functions *in vitro* but not *in vivo*.

One interpretation of these data is that NF-AT is synthesized but sequestered or compartmentalized in the cell, and when the cells are disrupted some transcriptionally active NF-AT is formed. To test this, we prepared nuclear and cytoplasmic extracts by a method that separates the two fractions more rapidly than our *in vitro* transcription extract protocol¹⁹. In these nuclear extracts prepared from stimulated/FK506-treated cells, NF-AT binding activity is substantially reduced and is not observed in the cytoplasmic fractions (Fig. 2a, lanes 3–6). Remarkably, binding activity was completely reconstituted by mixing nuclear extracts from stimulated/FK506-treated cells with cytoplasmic fractions from non-stimulated, or stimulated/FK506-treated cells, neither of which have NF-AT binding activity (Fig. 2a, lanes 7 and 9). Although the electrophoretic mobility of the reconstituted DNA-protein complex is slightly faster than the characteristic mobility of the native NF-AT complex, the DNA-binding specificity is the same (Fig. 2b, lanes 4–9). Nuclear extracts from non-stimulated cells are not complemented by any of the cytoplasmic extracts (Fig. 2b, lanes 1–3), suggesting that stimulation of the cells is essential for synthesis of the nuclear component of NF-AT.

We used rapamycin as a control for nonspecific effects of FK506. Rapamycin is a structural analogue of FK506, which also mimics a twisted leucyl-prolyl amide bond, binds FK-BP, and inhibits isomerase activity^{12,13,20}. Although rapamycin inhibits isomerase activity, it antagonizes the actions of FK506 on NF-AT-directed transcription^{13,17} and IL-2 mRNA levels^{21,22}. Rapamycin did not block translocation of the cytoplasmic component of NF-AT to the nucleus after activation (Fig. 2a, lane 10). This is consistent with its failure to block NF-AT binding activity and NF-AT-directed transcription¹⁷.

The effects of FK506 on NF-AT formation were also tested in a murine antigen-specific T-cell hybridoma, JK12/90.1 (ref. 23). Nuclear extracts from stimulated/FK506-treated JK12/90.1 cells have virtually no NF-AT, like Jurkat nuclear extracts (Fig. 2c, lanes 3 and 6). Furthermore, complementation of nuclear extracts from stimulated/FK506-treated Jurkat or JK12/90.1 cells with cytoplasmic extracts from Jurkat or JK12/90.1 cells reconstituted NF-AT binding activity (Fig. 2c, lanes 4–5 and 7–8). Interestingly, the electrophoretic mobility of the NF-AT complex reconstituted with JK12/90.1 cytoplasmic extracts has the same mobility as the native complex. These differences in mobility between cytoplasmic extracts from JK12/90.1 and Jurkat cells may be attributable to protease susceptibility, or the absence of an additional subunit in Jurkat cytoplasmic extracts. In any case, NF-AT binding activity can be reconstituted from extracts of both murine and human T cells.

We also tested the effects of cyclosporin A on nuclear association of NF-AT: it completely blocks NF-AT-directed transcription in T cells, and nuclear extracts of cells stimulated in the presence of cyclosporin A have less NF-AT binding activity than stimulated controls¹⁶. Mixing nuclear extracts from stimulated cyclosporin A-treated cells with cytoplasmic extracts from

the same cells or non-stimulated cells reconstitutes NF-AT binding activity (Fig. 2d, lanes 4–6). Again, non-stimulated nuclear extracts cannot be complemented by any cytoplasmic extract (Fig. 2d, lanes 1–3) so these results suggest that cyclosporin A and FK506 both block the translocation of a pre-existing cytoplasmic component which constitutes part of the NF-AT DNA-binding complex.

As FK506 and cyclosporin A affect events requiring Ca^{2+} mobilization but not those induced by protein kinase C (refs 17, 24–26), we tested the contribution of Ca^{2+} mobilization in the formation of the NF-AT complex. Nuclear extracts from PMA-treated cells reconstitute NF-AT (Fig. 3a, lanes 3–5), but those from ionomycin-treated cells result in little reconstitution (Fig. 3a, lanes 6–8). Furthermore, the cytosol from ionomycin-treated cells is less effective at reconstituting NF-AT than cytosol from either non-stimulated or PMA-stimulated cells (Fig. 3a, lanes 9–11). Nuclear extracts from cells treated with either PMA alone or ionomycin alone have 10- to 50-fold less NF-AT than reconstituted extracts (Fig. 3a: compare lanes 1 and 2 with lane 10). These data are consistent with a model in which Ca^{2+} signals

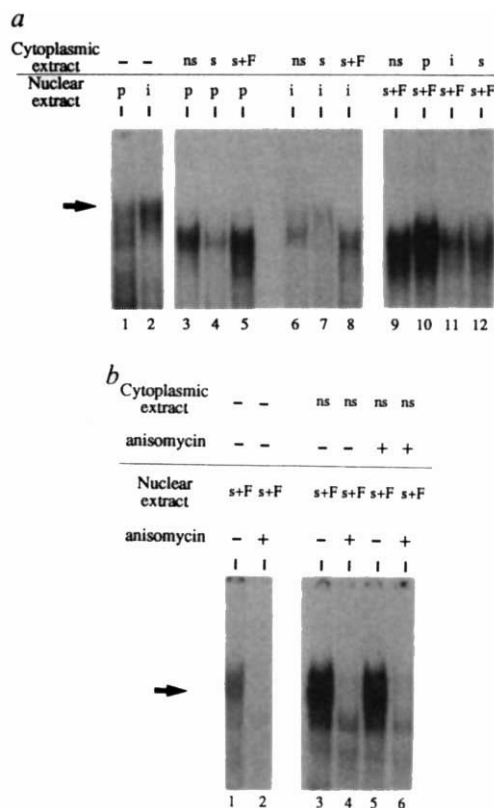


FIG. 3 The nuclear component of NF-AT is induced by PMA while calcium mediated signals allow translocation of the pre-existing cytoplasmic subunit of NF-AT. **a**, Lanes 1 and 2, gel mobility shift assay using nuclear extracts (10 μg) from PMA-stimulated (p) and ionomycin-stimulated (i) cells. In lanes 3–8, complementation of nuclear extracts (5 μg) from PMA-stimulated and ionomycin-stimulated cells with cytoplasmic extracts (5 μg) from non-stimulated (ns), stimulated (s), and stimulated/FK506-treated (s+F) cells. Stimulated/FK506-treated (s+F) nuclear extracts (5 μg) were complemented with cytoplasmic extracts (5 μg) from: lane 9, non-stimulated (ns); lane 10, PMA-stimulated (p); lane 11, ionomycin-stimulated (i); and lane 12, stimulated (s) cells. The arrows indicate the migration of the NF-AT complex. **b**, Lanes 1 and 2, gel mobility shift assay using nuclear extracts (10 μg) from stimulated/FK506-treated (s+F) cells in the presence (+) or absence (-) of 100 μM anisomycin. Lanes 3–6, complementation of nuclear extracts from stimulated/FK506-treated cells (with or without anisomycin pretreatment) with cytoplasmic extracts prepared from non-stimulated cells treated with or without anisomycin. NFATZ Jurkat cells were pretreated with 100 μM anisomycin (Sigma), an inhibitor of protein synthesis, for 30 min before stimulating the cells. Conditions for activating the cells and preparing nuclear and cytoplasmic extracts are described in Fig. 2.

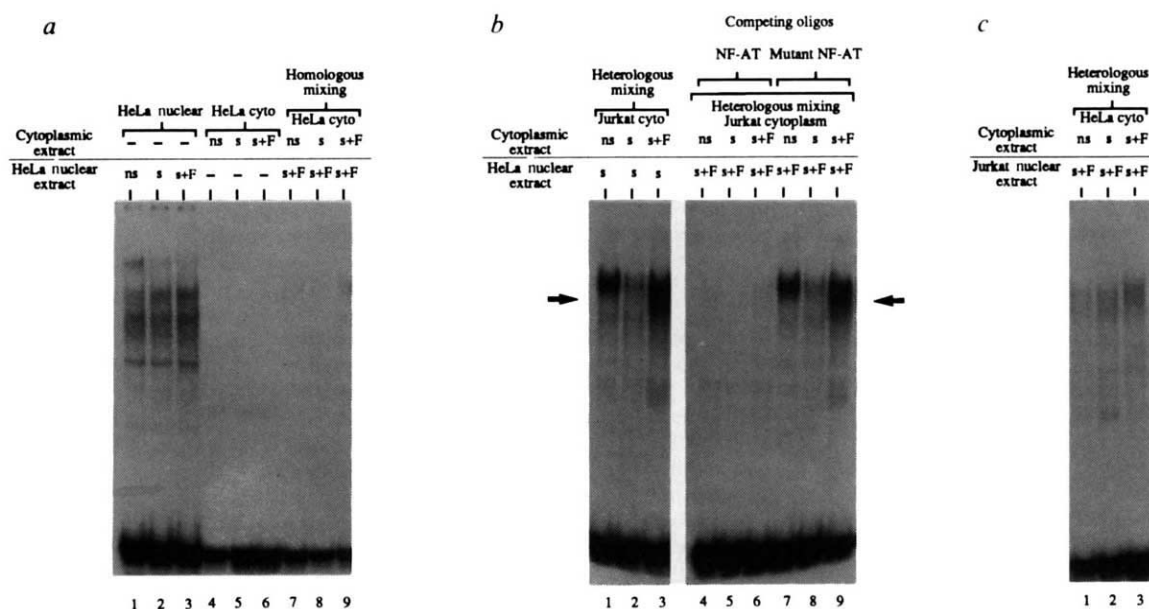


FIG. 4 The nuclear component of NF-AT is present in HeLa cells and can be complemented by Jurkat cytoplasm, but not by HeLa cell cytoplasm, to reconstitute NF-AT binding activity. *a*, Lanes 1–6, gel mobility shift assay using HeLa nuclear (10 μ g) and cytoplasmic (cyto; 10 μ g) extracts from non-stimulated (ns), stimulated (s), and stimulated/FK506-treated (s+F) cells do not form a NF-AT protein–DNA complex. In lanes 7–9, homologous mixing of nuclear extracts (5 μ g) from stimulated/FK506-treated (s+F) HeLa cells with HeLa cytoplasmic extracts (5 μ g) does not reconstitute NF-AT. *b*, NF-AT binding activity is reconstituted with HeLa nuclear and Jurkat cytoplasmic extracts. Nuclear extracts (5 μ g) from stimulated/FK506-treated HeLa cells complemented by cytoplasmic extracts (5 μ g) from: lane 1, non-stimulated (ns); and lane 3, stimulated/FK506-treated (s+F) Jurkat

cells. In lanes 4–9, reconstituted NF-AT binding complex demonstrates DNA-binding specificity when competed by 10 ng of unlabelled NF-AT or mutant NF-AT oligonucleotides (oligos). *c*, Lanes 1–3, heterologous mixing of Jurkat nuclear extracts (5 μ g) from stimulated/FK506-treated (s+F) with HeLa cytoplasmic extracts (5 μ g) does not reconstitute NF-AT binding activity.

METHODS. HeLa S3 cells were grown in spinner flasks at 37 °C in S-MEM (Gibco-BRL) supplemented with 5% fetal calf serum, penicillin (100 U ml⁻¹), and 100 μ g ml⁻¹ of streptomycin. HeLa S3 were stimulated, nuclear and cytoplasmic extracts were prepared, and gel mobility shift assays were carried out under conditions identical to those described in Fig. 2.

a pre-existing cytoplasmic subunit of NF-AT to associate with to the nucleus and complement the PMA-induced nuclear component of NF-AT.

To investigate whether the cytoplasmic component of NF-AT pre-exists and the nuclear component has to be synthesized, we prepared nuclear extracts from stimulated/FK506-treated cells and cytoplasmic extracts from non-stimulated cells in the presence or absence of 100 μ M anisomycin, which blocks 98% of protein synthesis in Jurkat cells¹⁵. Mixing nuclear extracts from anisomycin-treated cells with nontreated cytoplasmic extracts failed to reconstitute NF-AT binding activity (Fig. 3*b*, lane 2), although addition of cytoplasmic extracts from anisomycin-treated, non-stimulated cells to stimulated/FK506-treated nuclear extracts did (Fig. 3*b*, lane 5).

Despite the fact that the actions of cyclosporin A and FK506 are tissue-specific, their binding proteins are ubiquitous^{27–31}. This could be explained if the drug-isomerase complex acted on a T-cell-specific molecule. HeLa nuclear and cytoplasmic extracts do not contain NF-AT (Ref. 15) and homologous mixing of nuclear and cytoplasmic extracts from HeLa cells fails to reconstitute NF-AT binding activity (Fig. 4*a*, lanes 1–9), although heterologous mixing of Jurkat cytoplasmic extracts with nuclear extracts from HeLa cells can (Fig. 4*b*, lanes 1–3). Furthermore, the reconstituted NF-AT binding activity is specific, as demonstrated by oligonucleotide competition (Fig. 4*b*, lanes 4–9). These results suggest that the nuclear component of NF-AT is present in HeLa cells. In contrast, HeLa cell cytoplasmic extracts cannot reconstitute NF-AT binding activity when mixed with nuclear extracts from stimulated/FK506-treated Jurkat cells (Fig. 4*c*, lanes 1–3), implying that the cytoplasmic component is T cell-specific but the nuclear component of NF-AT is not.

On the basis of our results, we propose a model for the action of FK506 and cyclosporin A in which an inhibitory complex formed between the drug and the isomerase blocks nuclear translocation of a pre-existing, T cell-specific cytoplasmic component of NF-AT by disrupting a calcium-dependent signalling step close to NF-AT formation. The alternative possibility, that the drug-isomerase complex forms a stable association with NF-AT, is unlikely as we find no evidence for this association (M. Flanagan and G. Crabtree, unpublished results). In either case, defining the molecular mechanism of action of FK506 and cyclosporin A should lead to more effective design of immunosuppressants and to an understanding of an essential regulatory step in T-cell activation. □

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Effects of the *steel* gene product on mouse primordial germ cells in culture

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MUTATIONS at the *steel* (*sl*) and *dominant white spotting* (*W*) loci in the mouse affect primordial germ cells (PGC), melanoblasts and haemopoietic stem cells¹. The *W* gene encodes a cell-surface receptor of the tyrosine kinase family^{2,3}, the proto-oncogene *c-kit*. *In situ* analysis has shown *c-kit* messenger RNA expression in PGC in the early genital ridges⁴. The *Sl* gene encodes the ligand for this receptor, a peptide growth factor, called here stem cell factor (SCF)^{5–7}. SCF mRNA is expressed in many regions of the early mouse embryo, including the areas of migration of these cell types⁸. It is important now to identify the role of the *Sl-W* interaction in the development of these migratory embryonic stem cell populations. Using an *in vitro* assay system⁹, we show that SCF increases both the overall numbers and colony sizes of migratory PGC isolated from wild-type mouse embryos, and cultured on irradiated feeder layers of STO cells (a mouse embryonic fibroblast line). In the absence of feeder cells, SCF causes a large increase in the initial survival and apparent motility of PGC in culture. But labelling with bromodeoxyuridine shows that SCF is not, by itself, a mitogen for PGC. SCF does not exert a chemotrophic effect on PGC in *in vitro* assays. These results suggest that SCF *in vivo* is an essential requirement for PGC survival. This demonstrates the control of the early germ-line population by a specific trophic factor.

SCF was first tested at concentrations between 10 and 100 ng ml⁻¹ for its effect on overall numbers of PGC taken from early (8.5-days postcoitum (d.p.c.)) or late (10.5-d.p.c.) in their migratory period, and cultured as described previously⁹ on feeder layers of STO fibroblasts. In each case the overall numbers of PGC were increased by SCF after 3–5 days in culture (Fig. 1 and Table 1). Maximum effect was seen with doses between 30 and 40 ng ml⁻¹ in serum-free medium, and so this concentration range was used thereafter.

After about 3 days in culture on STO cells, PGC form discrete colonies (Fig. 2a). We counted both the numbers of these, and their sizes (as measured by the number of PGC) in the presence or absence of SCF. In two separate experiments SCF increased

TABLE 1 Overall numbers of 8.5- and 10.5-d.p.c. PGC cultured on STO cells are increased by SCF

	Day 1	Day 5
8.5-d.p.c. PGC in SF-1	32.7 (2.7)	322.0 (40.4)
8.5-d.p.c. PGC in SCF	38.6 (4.8)	537.0 (83.7)
P value from unpaired <i>t</i> -test	0.1–0.375	0.025–0.05
	Day 1	Day 3
10.5-d.p.c. PGC in SF-1	157.6 (24.9)	171.5 (9.6)
10.5-d.p.c. PGC in SCF	179.5 (13.0)	256.7 (3.1)
P value from unpaired <i>t</i> -test	0.1–0.375	0.0005

Mean PGC numbers were compared using unpaired *t*-tests.

the numbers and sizes of colonies after both 3 and 5 days in culture (Fig. 2b). Labelled bromodeoxyuridine (BRDU) analysis (Fig. 2c) showed that the division rate of PGC did not alter throughout 4 days of culture, and was not affected by SCF. As SCF does not increase the rate of division, it must act by increasing the numbers of PGC that survive and proliferate. This does not exclude the possibility that SCF can also stimulate proliferation in combination with other growth factors, as is the case with haemopoietic stem cells^{6,10,11}.

PGC were also cultured in the absence of STO cells, directly on plastic. These cultures contain contaminating somatic cells from the migratory path, which are known to release SCF *in vivo*⁸. Despite this, added soluble SCF had a large effect on initial survival and motility of 10.5-d.p.c. PGC (Fig. 2d). But this effect was only sustained for 48 h, and there was no increase in overall PGC numbers as seen on STO cells. This may be due to a requirement for an additional factor provided by STO cells, or for the presentation of SCF in a membrane-bound form by STO cells. The latter possibility is suggested by the results of Dolci *et al.*¹².

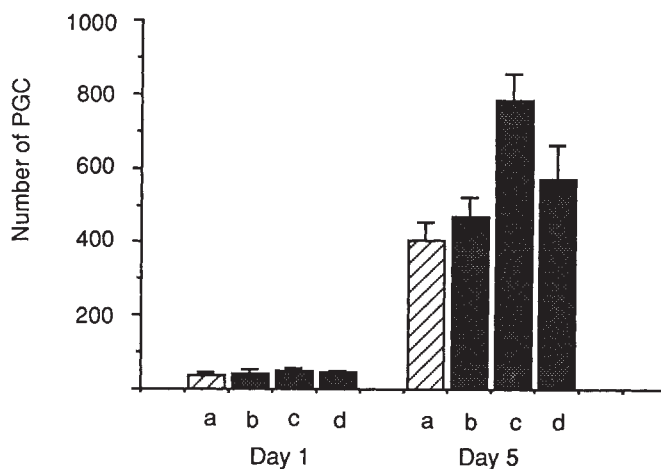


FIG. 1 PGC were removed from 8.5- or 10.5-d.p.c. MF1 embryos, and cultured on irradiated STO feeder cell layers in serum-free medium (SF-1; Northumbria Biologicals). Cultures were fixed after 1, 3 or 5 days and PGC counted by first staining them with alkaline phosphatase. Five replicate samples were counted in each case, and results expressed as means with standard errors. A sample experiment is shown in which 8.5-d.p.c. PGC were cultured in different concentrations of SCF. Peak response was seen with 37.5 ng ml⁻¹, and this concentration was used thereafter. Four duplicate experiments were done with 8.5-d.p.c., and three with 10.5-d.p.c. PGC. Overall means from these experiments, plus standard errors of the means are shown in Table 1. a, SF-1 medium; b, 18.8 ng ml⁻¹ SCF; c, 37.5 ng ml⁻¹ SCF; d, 75 ng ml⁻¹ SCF.

FIG. 2 *a*, Colonies of PGC formed after 3 days' culture on STO cells. PGC in colonies were counted at 3 and 5 days of culture. The colony size distribution for one 3-day experiment is shown in *b*. Total number of colonies in this experiment were 45 in serum-free medium and 79 in 37.5 ng ml^{-1} SCF. At 5 days, total colony numbers were 45 and 90, respectively. This experiment was repeated with near identical results. *c*, Incorporation of BRDU into PGC after a 4-h incubation period, during each day of culture on STO cells. Five assays were done at each time point, and the results expressed as percentage of PGC containing BRDU, together with standard errors. *d*, In the absence of STO cells, SCF stimulates initial survival of PGC. PGC (10.5-d.p.c) were cultured at high concentration (two embryo equivalents per microtitre well) with and without 37.5 ng ml^{-1} SCF. SCF increases the number of adherent PGC, and the number that show a motile phenotype¹⁴. Five duplicate cultures were counted on each day. Hatched bars, SF-1 medium only; dark bars, SF-1 medium + 37.5 ng ml^{-1} SCF.

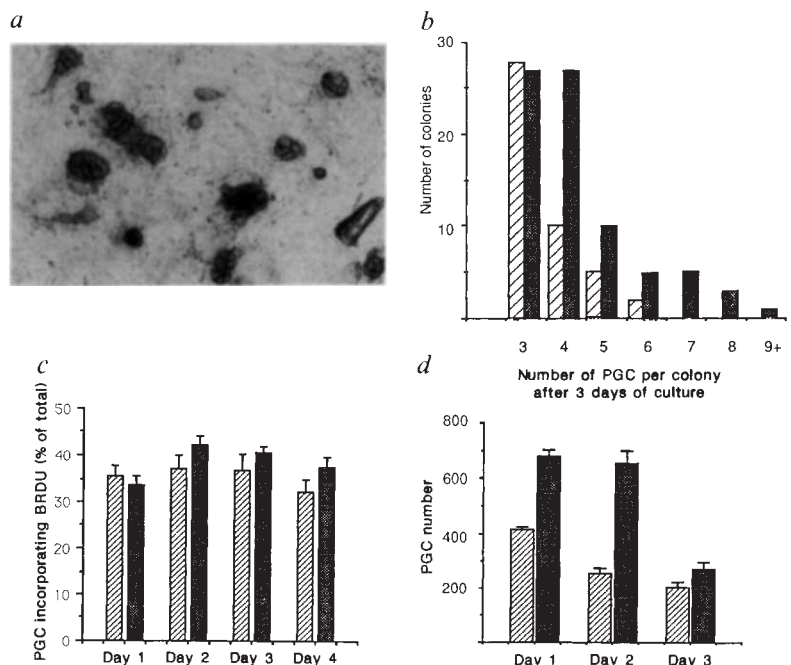
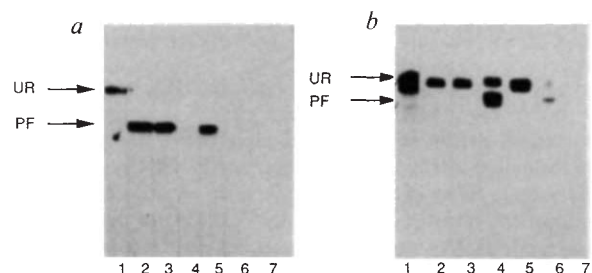


FIG. 3 STO cells express SCF but not *c-kit* mRNA. *a*, SCF protection assay; *b*, *c-kit* assay. UR, undigested riboprobe; PF, protected fragment. The lanes received the same loadings for each assay: 1, untreated riboprobe transcript; 2, 3T3 cells; 3, fetal liver; 4, fetal cerebellum; 5, STO cells; 6, FDCP-mix, undifferentiated; 7, FDCP-mix, differentiated. STO cells show a protected fragment of the expected size in the SCF assay, but not in the *c-kit* assay. STO cell RNA was prepared by lysis in 4 M guanidine isothiocyanate followed by centrifugation in caesium chloride. RNA probes were prepared from a 396-base pair (GP) fragment of a murine SCF cDNA and a 1,035-bp fragment of a murine SCF *c-kit* cDNA cloned into pGEM vectors following manufacturer's instructions (Promega). Following gel purification, $5-10 \times 10^5$ c.p.m. of probe was mixed with $20 \mu\text{g}$ cellular RNA precipitated and resuspended in $20 \mu\text{l}$ hybridization buffer, and protection assays carried out as in ref. 15, except that RNase digestion buffer was 0.3 M sodium acetate, 10 mM Tris-HCl, pH 7, 5 mM EDTA, $20 \mu\text{g ml}^{-1}$ RNase A and 50 units ml^{-1} RNase T1.



To eliminate the possibility that the effects seen on STO cells are mediated indirectly by the STO cells, we tested for the presence of *c-kit* mRNA in STO cells. As a positive control for the *c-kit* assay, we used RNA from FDCP-mix (for factor-dependent cells, Patterson-multipotent). These primitive haemopoietic stem cells express *c-kit* in the undifferentiated, but not in the differentiated state¹³. STO cells do not express *c-kit* mRNA, but do express SCF mRNA (Fig. 3). As the *c-kit* protein is the only known receptor for SCF, it is extremely unlikely that the effects of SCF in these assays is being mediated indirectly. The expression of SCF by STO cells suggests that the added SCF in these assays is enhancing a preexisting low-level signal, and would explain our original observation that STO cells promote the survival of PGC *in vitro*¹⁴.

To test whether SCF has a chemotropic effect on PGC, we used an assay⁹ in which the genital ridges (the normal target of PGC migration) exert a chemotropic effect on PGC in culture, whereas other tissues tested had no effect. SCF in concentrations of $10-50 \text{ ng ml}^{-1}$ did not exert a chemotropic effect in these assays, whereas medium conditioned by 10.5-d.p.c. genital ridges did so (data not shown). Soluble SCF is therefore unlikely to be the factor released by genital ridges in culture. SCF could control migration in other ways. In membrane-bound form its stimulation of motility (shown here) or control of adhesion could provide directional cues for the PGC.

So why do mutations at the *W* and *Sl* loci cause fewer PGC to colonize the genital ridges? These results suggest that when the supply of SCF to the migrating PGC population is reduced (as in *Sl*), or the response is disabled (as in *W*), then PGC fail to survive. Our chemotropic assays make it unlikely that the *W-Sl* interaction guides the PGC to their target, at least through a gradient of diffusible, soluble SCF. This conclusion is reinforced by the fact that in alleles of *W* and *Sl* that almost completely eliminate the PGC population, there is still a small number that reach the gonad. In normal development, a proportion of the PGC never reach the genital ridges, but colonize surrounding regions of the embryo, and die. Our results suggest that the mechanism whereby they die might be due to the absence of SCF in these tissues. Whether or not this is due to the ability of SCF to specifically suppress apoptosis in the germ-line cells is not yet known. But the existence of specific survival factors in spatially limiting supply could be a general mechanism for controlling the populations of migrating stem cell populations. □

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Requirement for mast cell growth factor for primordial germ cell survival in culture

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MAST-CELL growth factor (MGF) is encoded by the murine *steel* (*Sl*) locus and is a ligand for the tyrosine kinase receptor protein encoded by the proto-oncogene *c-kit* at the murine *dominant white spotting* (*W*) locus. Mutations at both these loci affect mast cells, primordial germ cells (PGCs), haemopoietic stem cells and melanocytes. In many *Sl* and *W* mutants, the rapid proliferation of PGC that normally occurs between day 7 and 13.5 of embryonic development fails to occur. As *c-kit* is expressed in PGCs^{1,2} while MGF is expressed in the surrounding mesenchyme^{2,3}, MGF might promote the proliferation of PGCs. Here we report that MGF is essential for PGC survival in culture, but does not stimulate PGC proliferation. Moreover, whereas both the transmembrane and soluble proteolytic cleavage forms of MGF stimulate mast-cell proliferation, soluble MGF has a relatively limited ability to support survival of PGCs in culture, thus explaining the sterility in mice carrying the *steel-dickie* (*Sl^d*) mutation, which encodes only a soluble form of MGF, and providing a functional role for a transmembrane growth factor.

The ability of MGF to promote survival and/or proliferation of PGCs was determined using a culture system in which both the type and amount of MGF present could be manipulated. Isolated pregonadal (8.5–10.5 days) PGCs survive <24 h in culture in the absence of a confluent monolayer of feeder cells⁴. PGCs do survive when cultured on feeder layers of STO or NIH-3T3 cells^{4–6} but not on feeder layers of CV-1 cells (Fig. 1a). The differences in PGC survival on STO and NIH-3T3 versus CV-1 feeder layers might be due to differential MGF expression by these feeder cell lines. STO and NIH-3T3 cells both express a 6.5-kilobase (kb) MGF messenger RNA and produce MGF that promotes the proliferation of mast cells, whereas CV-1 cells do neither (Fig. 1b; Table 1). CV-1 cells

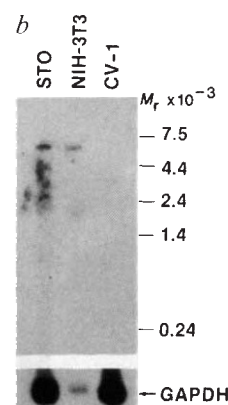
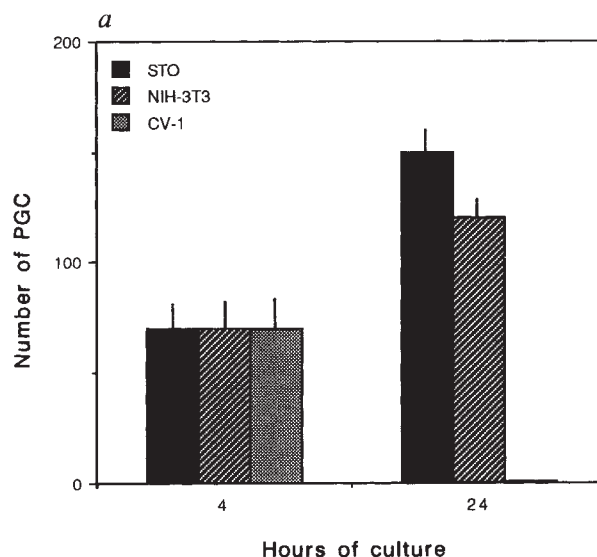


FIG. 1 a, Survival of PGCs on STO, NIH-3T3 or CV-1 feeder layers. The number of PGCs present after 4 and 24 h of culture. Bars represent the mean plus/minus the standard deviation of five replicate cultures. Each experiment was done four times. Solid bar, STO; hatched bar, NIH-3T3; dotted bar, CV-1. b, Northern analysis of MGF expression by feeder cell lines, STO cells, NIH-3T3 cells and CV-1 cells. RNA loading was assayed by probing for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

METHODS. STO cells and NIH-3T3 cells are mouse embryo-derived fibroblast cell lines. CV-1 cells are fibroblast-like cells derived from the kidney of a male African green monkey. Cells were grown in DMEM (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and either 10% FBS (STO and CV-1) or 10% calf serum (NIH-3T3 cells). Staged embryos were derived from timed matings of B6C3F1 animals as described⁴, and PGCs isolated from 8.5-day postcoitus (dpc) embryos by dissection of a fragment consisting of the caudal end of the primitive streak and the base of the allantoic rudiment⁶. These fragments were dissociated in 0.05% trypsin, 0.02% EDTA (Sigma) to yield a single-cell suspension of PGC and somatic cells. This cell suspension was plated onto confluent, irradiated, feeder layers as previously described⁴. PGC were maintained in feeder-layer culture in DMEM supplemented with 15% FBS and identified by alkaline phosphatase histochemistry^{4,6}. For northern analyses, confluent cell monolayers were washed with PBS, lysed with RNAzol (Cinna/Biotex) and total RNA prepared by the method of Chomczynski and Sacchi²². Poly(A)⁺ RNA was selected using a Fast Track kit (Invitrogen). Poly(A)⁺ RNA (2 µg) were run on a 1.5% agarose-formaldehyde gel, transferred to Nytran (Schleicher and Schuell) and baked. Northern blots were probed with a 2.0-kb *Sal*I fragment of an MGF cDNA clone (MGF-10), representing the entire MGF coding sequence¹⁵. The probe was ³²P-labelled using a Multiprime labelling kit (Amersham) and hybridizations were done according to Church and Gilbert²³. To quantitate RNA loading, blots were stripped and reprobed with a GAPDH cDNA probe²⁴.

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transfected with a construct encoding transmembrane MGF (CV-1-Wt) are able to stimulate mast-cell proliferation and support PGC survival for at least 3 days (Table 1; Fig. 2a). Taken together these data strongly suggest that MGF mediates PGC survival in culture.

Although MGF is required for survival it is not sufficient for proliferation of PGCs in culture. PGCs survive and proliferate on feeder layers of STO cells (Fig. 2b and refs 4–6). On NIH-3T3

cells, PGCs survive for up to 5 days, but their numbers gradually decline and their proliferation rate, assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation, is about 50% that of PGCs cultured on STO cells (Fig. 2b; data not shown). Addition of a recombinant form of MGF (rMGF) lacking the transmembrane domain and cytoplasmic tail has no effect on PGC proliferation on NIH-3T3 cells, despite its ability to stimulate mast-cell proliferation (Fig. 2b). Similarly, Godin *et al.*⁷ conclude that added soluble factor does not stimulate proliferation of PGCs cultured on STO cells. Apparently, PGC proliferation on STO cells is not simply due to higher levels of soluble MGF. STO cells either produce higher levels of transmembrane MGF, a unique form of MGF or, in addition to MGF, another factor which is able to stimulate PGC proliferation. Addition of STO cell-conditioned medium to NIH-3T3 cells stimulated PGC proliferation suggesting that STO cells produce, in addition to MGF, a soluble PGC mitogen (data not shown).

Many lethal *SI* mutants are completely deleted for MGF coding sequences^{8–10}. By contrast, the viable, but sterile, *SI^d* mutant is deleted only for sequences encoding the MGF cytoplasmic tail and transmembrane domain^{11,12}. In *SI/SI^d* mice, PGC form and migrate, but the few PGC that colonize the gonad anlagen do not survive¹³. As *SI^d* can produce only a soluble form of MGF, soluble MGF may be unable to support long-term survival of PGC. To test this idea, we compared PGC survival on CV-1 cells transfected with a construct encoding a truncated MGF identical to that encoded by the *SI^d* allele (CV-1-*SI^d*) and CV-1 cells transfected with the transmembrane MGF (CV-1-Wt). As CV-1 cells seem unable to cleave transmembrane MGF to the soluble form (D.E.W., unpublished observations) CV-1-Wt probably produce only transmembrane MGF whereas CV-1-*SI^d* cells produce only soluble MGF. CV-1-*SI^d* supported PGC survival to 70% of CV-1-Wt after 1 day ($P < 0.005$) and 40% of CV-1-Wt after 3 days ($P < 0.038$; Fig. 2a), despite the fact that

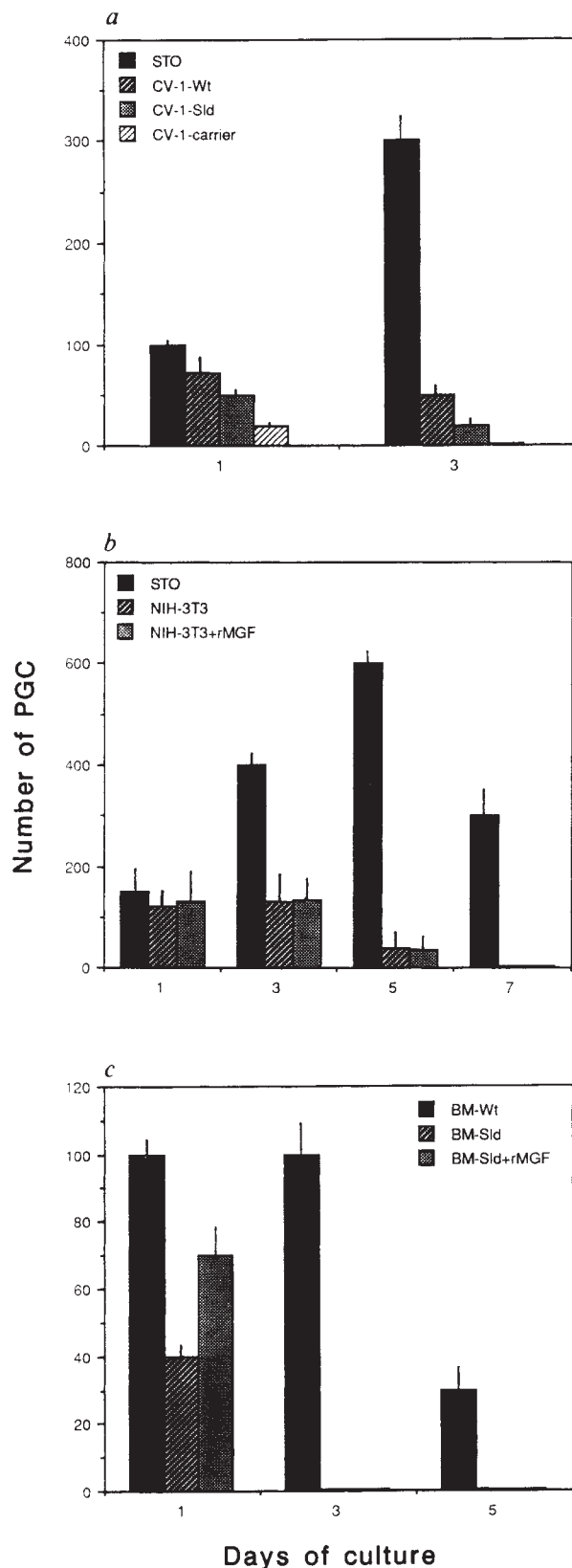


FIG. 2 Analysis of the survival and proliferation of PGCs in culture. *a*, The number of PGCs present on days 1 and 3 of culture on STO cells and CV-1 cells transfected with constructs encoding wild-type MGF (CV-1-Wt), *SI^d* MGF (CV-1-*SI^d*), or carrier DNA. This experiment was repeated four times. MGF expression was assayed as previously described¹⁴ using a mast cell line, MC-6, which is responsive to MGF (see Table 1). *b*, The number of PGC present at days 1, 3, 5 and 7 of culture on STO cells, NIH-3T3 cells and NIH-3T3 cells to which 100 ng ml⁻¹ rMGF was added. This experiment was repeated three times. *c*, The number of PGC present after 1, 3 and 5 days of culture on bone marrow stromal cell lines derived from wild-type (BM-Wt) and *SI/SI^d* (BM-*SI^d*) mice, and BM-*SI^d* cells to which 100 ng ml⁻¹ rMGF was added. Bars represent the mean $\pm$ the standard deviation of five replicate cultures. This experiment was repeated four times.

METHODS. Brains were taken from wild-type (C57BL/6J) or *SI/SI^d* (DBA) animals. Total RNA was prepared as above using RNazol (Cinna/Biotec)²². Each RNA was used as a template to synthesize single-stranded cDNA with the Copy Kit (Invitrogen). These cDNAs were used as a template in a polymerase chain reaction (PCR) with the amplimers: 5'-CGGAGTCGCC-ACACCGCTGC-3' and 5'-GTCACTTGAGACAGAATGGG-3' (400 ng each) with the following conditions: 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. Each reaction product was gel-purified, kinased, filled in with T4 polymerase and ligated into Bluescript (Stratagene). Each clone was sequenced to verify authenticity; Wt, 956 base pairs (bp) and *SI^d*, 782 bp. MGF was expressed from the SV40 early promoter vector pSG5 (Stratagene). An *XhoI* linker was placed in the pSG5 *BamHI* site, and the *XhoI*-*BamHI* polylinker sites of pBluescript SK (Stratagene) transferred into the pSG5 *XhoI* and *BglII* sites. *XhoI*-*BamHI* fragments from PCR reactions of the Wt and *SI^d* alleles were then cloned into these expanded polylinkers. CV-1 cells on 10-cm dishes were transfected with 20 μ g expression vector by the calcium phosphate technique²⁵. The cells were shocked with 15% glycerol 18 h after the addition of precipitate. Bone marrow stromal cells were cultured in McCoy's 5A medium (Gibco) supplemented with 10% heat-inactivated FBS as previously described^{14,26}. PGCs were isolated as previously described (see above) and plated onto confluent monolayers of stromal cells that had been irradiated with 5,000 rads to induce quiescence. Cultures were maintained in DMEM plus 15% FBS (as described earlier). The rMGF was produced as previously described¹⁵ and used over a concentration range of 0.001–1 μ g ml⁻¹.

TABLE 1 Proliferation of the MGF-dependent mast cell line MC-6 on established cell lines and transfected CV-1 cells

Cell line	³ H-thymidine incorporation (c.p.m.)
STO	6,728 ± 2,413*
NIH-3T3	9,849 ± 3,839†
CV-1	2,064 ± 138
CV-1 WT MGF	7,552 ± 575†
CV-1 <i>Sl^d</i> MGF	13,244 ± 1,917†
CV-1 + carrier	2,365 ± 492

Proliferation assays were essentially carried out as previously described¹⁴. Briefly, 20,000 feeder cells were plated into microtitre wells and irradiated with 5,000 rads to induce quiescence. After 24 h, 10,000 MC-6 cells were added to each well and after a further 24 h the cultures were pulsed for 4–5 h with 1 µCi [³H]thymidine. Wells were collected on fibreglass filters with an automated collector and incorporated [³H]thymidine counted by liquid scintillation spectrometry. This experiment was repeated four times with six replicates per treatment.

* $P \leq 0.05$ versus CV-1 cells or CV-1 + carrier.

† $P \leq 0.001$ versus CV-1 cells or CV-1 + carrier.

the total MGF produced by CV-1-*Sl^d* (assayed by mast cell proliferation) was higher than that of CV-1-Wt (Table 1). We also compared PGC survival on *Sl/Sl^d* (BM-*Sl^d*) and congenic wild-type (BM-Wt) bone marrow-derived stromal cell lines. BM-*Sl^d* supported PGC survival to 40% of BM-Wt after 1 day, but did not support subsequent PGC survival (Fig. 2c). When recombinant MGF (rMGF) was added to PGCs cultured on BM-*Sl^d*, PGC survival of over one day improved to 70% of BM-Wt, but subsequent PGC survival was unaffected (Fig. 2c). When rMGF was added to PGCs cultured on NIH-3T3 or STO cells, PGC survival was not enhanced (Fig. 2b and data not shown). Presumably, in the culture conditions used in this study, the amount of MGF produced by NIH-3T3 and STO cells is sufficiently high to support maximal PGC survival in the absence of added rMGF (see Fig. 1b and Table 1). Soluble MGF, such as that produced by *Sl/Sl^d* cells, is able to support only limited PGC survival and is unable to support long-term survival seen when PGC are cultured on wild-type cells producing both transmembrane and soluble MGF. In a similar study, Godin *et al.*⁷ report that soluble factor is able to enhance initial survival (>48 h) but does not promote long-term survival of PGC cultured in the absence of STO cells.

The reduced ability of soluble MGF to support long-term survival may reflect a requirement for a localized, high concentration of MGF, for a particular conformation of MGF, for a role of MGF in promoting cell adhesion, or for an extended ligand-receptor interaction precluded by internalizable, soluble MGF. That transmembrane MGF is more effective in supporting PGC survival could provide (1) a mechanism both for the haptotactic guidance of PGCs to the gonad anlagen as well as for strict regulation of PGC proliferation and differentiation in the embryo and (2) a possible explanation for the sterility found in *Sl/Sl^d* mice. The viability, but sterility and lack of pigment, of *Sl/Sl^d* mice suggests that the haemopoietic lineages can be maintained to some extent by soluble factor, whereas PGCs and melanoblasts cannot. The activity of soluble factor on mast cells and primitive haemopoietic progenitors *in vitro*^{14–16} and on mast cells *in vivo*⁹ is consistent with this idea.

Binding of tyrosine kinase receptors, such as *c-kit*, by their cognate ligands usually leads to activation of the kinase domain and transduction of signals that lead to cellular proliferation^{17–19}. The MGF/*c-kit* complex mediates a proliferation response in mast cells^{14,15,20} and, in combination with other factors, proliferation of primitive haemopoietic progenitors^{11,15,16,20,21}. The data presented here and by Godin *et al.*⁷ provide the first evidence of growth factor action on mouse PGCs and suggest a role for MGF in mediating a PGC survival signal rather than a proliferation signal. □

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Carboxy-terminal truncation activates *glp-1* protein to specify vulval fates in *Caenorhabditis elegans*

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THE *glp-1* and *lin-12* genes encode homologous transmembrane proteins^{1,2} that may act as receptors for cell interactions during development^{3,4}. The *glp-1* product is required for induction of germ-line proliferation and for embryogenesis^{3,5}. By contrast, *lin-12* mediates somatic cell interactions, including those between the precursor cells that form the vulval hypodermis (VPCs)⁶. Here we analyse an unusual allele of *glp-1*, *glp-1(q35)*, which displays a semidominant multivulva phenotype (Muv), as well as the typical recessive, loss-of-function *Glp* phenotypes (sterility and embryonic lethality)³. We find that the effects of *glp-1(q35)* on VPC development mimic those of dominant *lin-12* mutations, even in the absence of *lin-12* activity. The *glp-1(q35)* gene bears a nonsense mutation predicted to eliminate the 122 C-terminal amino acids, including a ProGluSerThr (PEST) sequence thought to destabilize proteins. We suggest that the carboxy terminus bears a negative regulatory domain which normally inactivates *glp-1* in the VPCs. We propose that inappropriate *glp-1(q35)* activity can substitute for *lin-12* to determine vulval fate, perhaps by driving the VPCs to proliferate.

During wild-type development, three VPCs (P5.p, P6.p, P7.p) generate 22 descendants that form the vulval hypodermis (Figs 1 and 2a). Several genes are known that, when mutant, interfere with this process (reviewed in ref. 7). One of these is *lin-12*: animals with elevated *lin-12* activity (*lin-12(d)*) are Muv because all six VPCs adopt the VH2 fate⁶. The *glp-1* and *lin-12* genes

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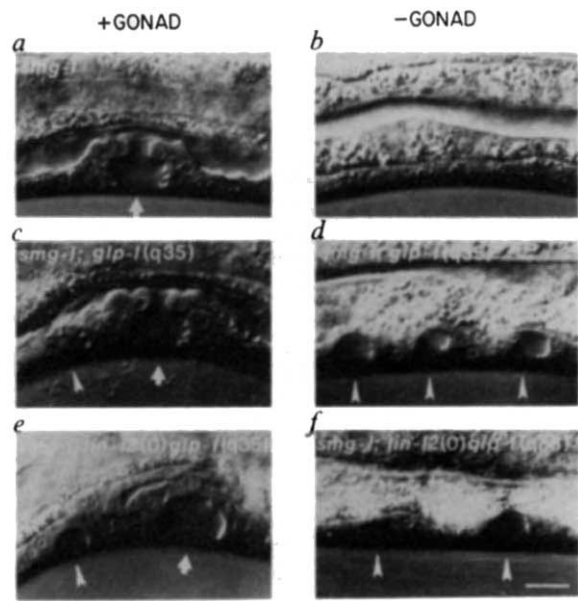


FIG. 1 Vulval phenotypes. Nomarski differential interference contrast (DIC) photomicrographs of fourth larval stage hermaphrodites. *a, c, e*, Intact animals that retain their gonad (+GONAD); *b, d, f*, animals that have had their gonad destroyed in early L1 (-GONAD). *a* and *b*, *smg-1*; *c* and *d*, *smg-1; glp-1(q35)*; *e* and *f*, *smg-1; lin-12(0)glp-1(q35)*. An arrow indicates the normal, AC-dependent vulval invagination; arrowheads indicate pseudovulval invaginations. Animals in *c, d, e* and *f* had additional pseudovulvae not shown in the figure. Scale bar ~10 μ m.

METHODS. Alleles used were the loss-of-function allele *smg-1(r861)* I (ref. 10), the putative null allele *lin-12(n941)* II (ref. 6) and *glp-1(q35)* III. *smg-1;glp-1(q35)* III animals were maintained as a homozygous stock; *smg-1;lin-12(0)glp-1(q35)* animals from *smg-1;lin-12(0)glp-1(q35)/dpy-19(e1259)unc-69(e587)* mothers were identified by fate transformations typical of *lin-12(0)*, such as an extra AC, or posterior ventral coelomocytes⁶. Nomarski DIC microscopy was performed as described¹⁸. Animals were maintained by standard laboratory techniques¹⁹ at 20 °C (operated animals) and 23 °C (all others). To destroy the gonad, the somatic gonadal precursor cells Z1 and Z4 were killed during the first larval stage of hermaphrodites that had been anesthetized in 20 mM sodium azide/1 $\times$ M9. Laser microsurgery was performed as described²⁰ using a Zeiss axioskop microscope, VSL-337 nitrogen laser, and DLM-110 dye laser module. Animals were examined in the third larval stage to ensure that the gonad was absent, and that there was no other structural damage.

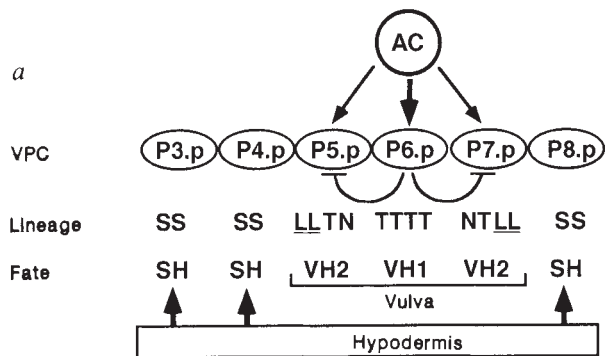


FIG. 2 *a*, Model for wild-type vulval development; see ref. 7 for review. Three (P5.p, P6.p, and P7.p) of six vulval precursor cells (VPCs) located in the ventral hypodermis divide three times and form the vulval hypodermis (VH1 (or 1°) and VH2 (or 2°) fates)¹⁸. The remaining three cells (P3.p, P4.p, and P8.p) divide once and fuse with the hypodermal syncytium (SH (or 3°) fate)¹⁶. Each VPC has the potential to adopt the VH1, VH2 or SH fate^{12,20,22}. In wild-type, however, the fate of each is invariant¹⁸. VH1 and VH2 fates are induced by the anchor cell (AC), a cell of the somatic gonad¹², whereas SH fate is promoted by a signal from the hypodermis (arrows)²¹. Although inductive signals are diagrammed as emanating directly from the AC and hypodermis, these signals may be indirect. The choice between VH1 and VH2 depends on the proximity of a VPC to the AC, and on a signal from the VH1 to the VH2 cell (bar)^{22,23}. VH1 and VH2 fates are distinguished by the axis of the third division and the adherence of the progeny cells to the ventral cuticle²². Axes are either transverse (T), or longitudinal (L); no third division is designated as N. Cell divisions that generate adherent daughters are underlined; if daughters are nonadherent, the division axis is not underlined. Cells that fuse with the syncytial hypodermis are denoted by an S. The VH1 fate is characterized by four nonadherent divisions (normally TTTT), whereas the VH2 fate is characterized by two adherent divisions and 1–2 non-adherent divisions (usually NTLL or LLTN). *b*, Vulval fates in *glp-1(q35)* and *lin-12* mutants. The VPC lineages of the following animals were obtained: (1) wild-type (N2 animals with a gonad (*smg-1*) animals behave like N2 (ref. 10); (2) *smg-1* animals in which the gonadal precursors (and hence the AC) were destroyed by laser microsurgery; (3, 4) *lin-12(d)/+* and (5) *lin-12(d)* animals that lack an AC owing to cellular transformation⁶; (6–12) *smg-1;glp-1(q35)* animals with a gonad; (13–17) *smg-1;glp-1(q35)* animals in which the gonadal precursors were destroyed by laser microsurgery; (18–23) *smg-1;lin-12(0)glp-1(q35)* animals with a gonad; (24–26) *smg-1;lin-12(0)glp-1(q35)* animals in which the gonadal precursors were destroyed. Division axes are as described in *a*. In addition, oblique (O) divisions were occasionally seen. Cells that divided, but in which the plane of division was not observed, are marked as D. Each line represents an individual VPC lineage. Asterisk, the AC was centered over P5.p; dagger, P9.p followed a vulval lineage: L⁺LL. METHODS. Alleles were the same as those listed in the legend to Fig. 1, as well as the dominant gain-of-function allele *lin-12(n952)* III⁶; *lin-12(d)* and *lin-12(d)/dpy-19lin-12(+)**unc-69* animals were distinguished by progeny testing. Lineages were performed as described in the legend to Fig. 1.

Expt	Geno	Anchor cell	VPC					
			P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
1	N2	+	SS	SS	LLTN	TTTT	NTLL	SS
2	<i>smg-1</i>	-	S	S	S	S	SS	S
3	<i>lin-12(d +)</i>	-	LLSL	LLLT	LLTN	LLTT	NTLL	LLLL
4			LLLL	LLLL	LLTT	NTLL	LOLL	SS
5	<i>lin-12(d d)</i>	-	SS	SS	LLLN	LLLN	NTLL	LLLL
6	<i>smg-1;</i>	+	LLLT	LLLL	LLTN	TTTT	NTLL	LLLL
7	<i>glp-1(q35)</i>		SS	SSSS	LLTN	TTTT	NTLL	LLNN
8			LNLT	LLLL	LLTN	TTTT	NTLL	SS
9			SS S	SS	LLTN	TTTT	NTLL	SS
10			LLLT	LLLL	LLTN	TTTT	NTLL	LLLN
11			SSLL	SSLL	LLTN	TTTT	NTLL	LLTL
12			SSLL	LLTN	TTTT	NTLL	LLLL	SS
13	<i>smg-1;</i>	-	S LL	SS	SNLL	LLNN	LLLL	SS
14	<i>glp-1(q35)</i>		S	S	SS	LLLL	LLLN	LLLL
15			SS	SS	LLNT	LLLT	LLLT	LLLL
16			S NL	SS	SNLL	LLLL	SS	LL S
17			SS	LL S	LTLL	LLLT	LLLN	LL S
18	<i>smg-1;</i>	+	SLLL	LLLO	LLTT	TTTT	TTTT	NTLL
19	<i>lin-12(0)</i>		SS	S SS	NNTL	TTTT	NTLL	NLLL
20	<i>glp-1(q35)</i>		LLLL	LLLL	LLTN	TTTT	TTLL	SS
21			SS S	SS	LLTN	TTTT	NTLL	LLLL
22			SS S	SS	LLTN	TTTT	TTLL	LL S
23			LLLL	LLLL	LLTD	TTTT	NTLL	LLLL
24	<i>smg-1;</i>	-	LLLL	LLLT	LLTL	LLLL	LLLL	S OL
25	<i>lin-12(0)</i>		SS	SSLT	LLLL	LLLL	LLLL	LLLT
26	<i>glp-1(q35)</i>		LLLL	SS S	LLLL	LLLL	LLTN	LLTN ⁺

encode similar proteins^{1,2}. Furthermore, *lin-12* and *glp-1* are redundant during embryogenesis⁸, and may be able to respond to the same signal from the gonadal anchor cell (AC)⁹. These observations raise the possibility that *glp-1(q35)* is Muv because it is mimicking *lin-12(d)*, so we have compared the effects of *glp-1(q35)* and *lin-12(d)* on vulval development.

Several observations indicate that the *glp-1(q35)* Muv phenotype results from increased or novel *glp-1* activity. First, this phenotype is dominant, whereas all *glp-1* loss-of-function mutations are recessive (Fig. 3a and b; ref. 3). Second, this class of mutation is rare (one GlpMuv per 10⁴ haploid genomes; ref. 3, and our unpublished results). Third, *glp-1(q35)* homozygotes are more Muv than *glp-1(q35)/glp-1(0)* or *glp-1(q35)/+* heterozygotes (Figure 3a). Fourth, the informational suppressor, *smg-1* (ref. 10), suppresses the *glp-1(q35)* loss-of-function Glp phenotypes, but enhances its Muv phenotype (Fig. 3b). Certain mutants are rescued by *smg* mutations, apparently by increasing the abundance of aberrant RNAs (R. Pulak and P. Anderson, personal communication). By analogy, *smg-1* is predicted to increase the amount of *glp-1(q35)* RNA, and consequently protein, thereby suppressing the loss-of-function phenotypes and enhancing the gain-of-function phenotype.

To investigate whether the *glp-1(q35)* fate transformations are similar to those of *lin-12(d)*, we examined VPC development in *smg-1;glp-1(q35)* double mutants (Figs 1c and 2b). The double mutant was used to enhance the Muv phenotype. Whereas VPC fates are not altered in *smg-1* mutants¹⁰, in *smg-1;glp-1(q35)*

homozygotes, P3.p, P4.p and P8.p often follow a VH2-like fate rather than an SH fate (for example, see Fig. 2b, rows 6 and 10). Although other P(3,4,8).p lineages are harder to characterize, they are never VH1-like. These fate transformations are reminiscent of weak *lin-12(d)* alleles (Fig. 2b, rows 3–5).

In addition to the ectopic pseudovulvae made by P(3,4,8).p, a normal vulva is always formed in *smg-1;glp-1(q35)* animals, indicating that the VPCs are still responsive to the anchor cell signal. This is especially apparent when the AC is mispositioned, and the vulva is shifted accordingly (Fig. 2b, row 12). Similarly, when an AC is present in *lin-12(d)* mutants, the VPCs respond to it¹¹.

We next asked whether the *glp-1(q35)* Muv phenotype, like that of *lin-12(d)*, is independent of the AC. To address this question we destroyed the gonadal precursor cells during the first larval stage, before the AC is born. Although no vulva develops in wild type¹² and *smg-1* animals lacking an AC (Figs 1b and 2b, row 2), pseudovulvae are still made in *smg-1;glp-1(q35)* animals without an AC (Figs 1d and 2b, rows 13–17). Thus, *glp-1(q35)* does not act in the gonad to promote pseudovulva formation, nor does *glp-1(q35)* require a signal from an AC or VH1 cell, as these cell types are not formed in ablated animals. Interestingly, P(5–7).p are more likely to follow a vulval fate in ablated animals than P(3,4,8).p (Fig. 2b, rows 13–17). This bias may reflect a difference between the cells themselves (for example, wild-type P(5–7).p versus P(3,4,8).p), or their environments. Alternatively, *glp-1(q35)* may affect

FIG. 3 Characterization of *glp-1(q35)* Muv and Glp phenotypes. a, Dosage studies with *glp-1(q35)/glp-1(q35)* and *glp-1(q35)/dpy-19glp-1(+)/unc-69* animals were obtained from *glp-1(q35)/dpy-19glp-1(+)/unc-69* mothers at 23 °C. Genotypes were identified by progeny testing; *glp-1(q35)/glp-1(0)* animals were obtained by mating *glp-1(q35)/dpy-19glp-1(+)/unc-69* hermaphrodites that had no more self-sperm with *glp-1(q46)/dpy-19glp-1(+)/unc-69him-5(e1490)* males. *glp-1(q46)* is null by genetic³ and molecular criteria: it carries a nonsense mutation in the extracellular domain (V. Kodoyianni *et al.*, manuscript in preparation). The success of each mating was assayed by comparing the number of XX and XO progeny, and the number of *glp-1(q35)/+* to *glp-1(q46)/+* F1 hermaphrodites. *glp-1(q35)/(q46)* animals were identified by their Glp phenotype. b, *smg-1* enhances the Muv phenotype and suppresses the Glp phenotypes of *glp-1(q35)*. *smg-1;glp-1(q35)* and *smg-1;glp-1(q35)/dpy-19glp-1(+)/unc-69* hermaphrodites from *smg-1;glp-1(q35)/dpy-19glp-1(+)/unc-69* mothers were identified by progeny testing at 23 °C.

* The number of pseudovulvae in young adult or fourth larval stage (L4) animals was determined by Nomarski DIC optics. We used these optics so that the effects of *smg-1* on vulval morphogenesis¹⁰ did not alter our assessment of the presence or absence of pseudovulvae.

† The percentage of Muv animals was calculated using the data from the preceding columns.

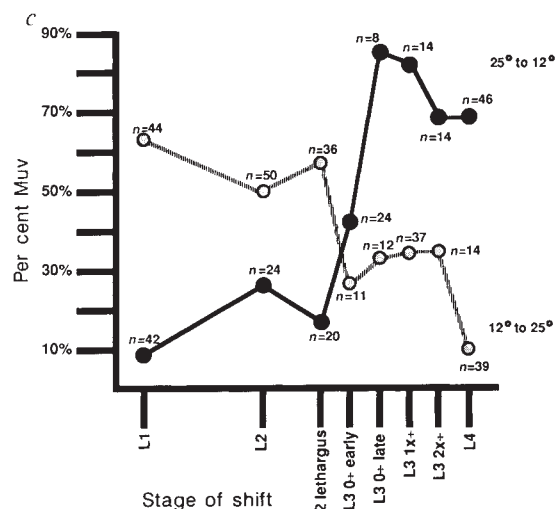
‡ Single L4 animals were picked to individual plates and allowed to lay eggs overnight. Animals without embryos were scored as Glp.

§ L4 hermaphrodites were picked to individual plates, and allowed to lay eggs. Animals were moved daily, 24 hours after an animal was removed, its progeny were scored as unhatched eggs, L1 larval lethals or viable.

NA, not applicable; 0, null; +, wild type.

c, *glp-1(q35)* activity is required at the time of VPC determination. The *glp-1(q35)* mutation is temperature-sensitive: animals raised at 25 °C are often Muv and fertile, whereas those grown at 12 °C are generally non-Muv and Glp. For the downshift, *glp-1(q35)/dpy-19glp-1(+)/unc-69* hermaphrodites were allowed to lay eggs for short periods of time (~1 h) at 25 °C. Animals were then removed, and the eggs allowed to develop at 25 °C until the stage indicated. Larvae were staged by Nomarski differential interference contrast (DIC) optics using standard criteria¹⁸. Larvae of the appropriate age were transferred to 12 °C plates, allowed to develop, and scored as adults. Homozygous *glp-1(q35)* animals were identified by the presence of meiotic germ cells in the distal arm of the gonad, and examined for pseudovulvae and eggs by Nomarski DIC microscopy as adults. Equivalent procedures were used for the upshift (12 °C to 25 °C). L1–L4, First through to the fourth larval stages; 0÷, 1x÷ and 2x÷ indicate the number of Pn.p cell divisions;

	Number of pseudovulvae*					%Muv†	%Glp‡	% Survival of progeny§
	0	1	2	3	4			
a Dosage								
<i>glp-1(+)</i>	60	0	0	0	0	0%	0%	98%
							(n=60)	(n=284)
<i>glp-1(q35)</i>	11	39	8	3	2	83%	94%	0%
							(n=70)	(n=9)
<i>glp-1(q35)/+</i>	47	16	2	0	0	28%	0%	98%
							(n=65)	(n=214)
<i>glp-1(q35)/glp-1(0)</i>	51	14	2	1	1	26%	100%	NA
							(n=69)	
b Effect of <i>smg-1</i>								
<i>smg-1;glp-1(+)</i>	60	0	0	0	0	0%	0%	98%
							(n=60)	(n=170)
<i>smg-1;glp-1(q35)</i>	4	13	20	19	3	93%	3%	25%
							(n=59)	(n=175)
<i>smg-1;glp-1(q35)/+</i>	27	22	11	0	0	55%	0%	99%
							(n=60)	(n=190)



early, Pn.p cells small and far apart; late, Pn.p cells larger and close together; filled circles, worms were shifted from 25 °C to 12 °C; open circles, worms were shifted from 12 °C to 25 °C; n is the number of *glp-1(q35)* animals assayed per timepoint.

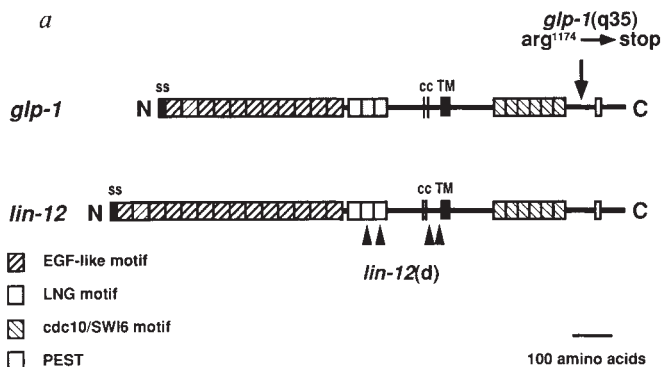


FIG. 4 *glp-1(q35)* is a nonsense mutation that removes the carboxy-terminal portion of the protein. **a**, The predicted proteins of *lin-12* and *glp-1* are shown for comparison^{1,2,24,25}. Both are transmembrane proteins with a signal sequence (ss) and membrane-spanning domain (TM); both carry three repeated motifs, one similar to epidermal growth factor (EGF-like), one found in *lin-12*, *Notch* and *glp-1* (LNG), and a third found in cell-cycle regulators and ankyrin (*cdc10/SWI6*). Also shown is the position of two cysteines conserved in *glp-1*, *lin-12* and *Notch* (CC) and a sequence proposed to act as a protein degradation signal (PEST). *glp-1* and *lin-12* are very closely linked on chromosome III (<0.02 centimorgans or ~18 kilobases¹⁻³). In *glp-1(q35)*, a CGA codon (encoding Arg 1,174) has been changed to a TGA stop codon (arrow). The approximate locations of the *lin-12(d)* mutations, all of which are missense²⁶, are indicated by arrowheads. **b**, The 122 amino acids predicted to be lost from the cytoplasmic domain of the *glp-1(q35)* protein are shown aligned with the corresponding region of *lin-12*. A strong PEST sequence encoded by both genes is underlined. Identities are shown by vertical lines, conservative changes by dots. The alignment is from ref. 2.

b

<i>glp-1</i> :	QQIVKSGHGA	KSGRQTVDNI	KRAGSRKTPT	SAASSRETNH	LTTPPSDGSF	SSPSP-HYYP	TTTSTPNRME
	I						
<i>lin-12</i> :	LHI-QHTHP	QPSRKVTTRAP	KKQTSRSKKE	SASNSRDSTH	LTTPPSDGSF	STPSQHFHMN	THHTPTSLN
							
<i>glp-1</i> :	-TSPEYMFNH	EMAPPVNA--	-----MWY	T-TPPPYQDP	NYRHVPNTA	FQNAEQMNGS	FCY
							
<i>lin-12</i> :	YLSPEYQTEA	GSSEAFQPC	GAFGNGEMWY	TRASTSYTQM	QNEPMTRYSE	PAHYF	

METHODS. The *glp-1(q35)* mutation was identified using a modification of the mismatch detection method²⁷. The *glp-1(q35)* allele was isolated from a recombinant DNA library constructed from *glp-1(q35)/glp-1(q35);qDp3* hermaphrodites [*qDp3* is *glp-1(+)*]. Genomic DNA was cleaved with *XhoI* ligated to EMBL3 lambda phage vector cut with *Sall*. The *glp-1(q35)* recombinants isolated this way contained ~2 kilobase pairs of 5' flanking sequences. The protocol described in ref. 27 was modified as follows: (1) recombinant DNAs were restricted to produce fragments of 100–800 base pairs in the region to be probed; (2) after hybridization, duplexes were

digested with 0.4 $\mu\text{g } \mu\text{l}^{-1}$ of RNase A (Sigma). We used a series of five probes (described in V. Kodoyianni *et al.*, manuscript in preparation) covering the entire gene and flanking sequences. Both *glp-1(+)* and *glp-1(q35)* clones were isolated, and the two classes of recombinants were distinguished by mismatch detection. The carboxy terminal PEST sequence was identified using the IBM PC Gene program of Intelligenetics, using the algorithm of Rogers *et al.*¹⁴ The PEST sequence of *glp-1* scored 10.8 (>5 considered 'good').

individual VPCs differently. This same phenomenon is observed in *lin-12(d)* mutants (Fig. 2b, rows 3–5; ref. 13, and data not shown).

The *glp-1(q35)* mutation is temperature-sensitive, which allowed us to determine when *glp-1(q35)* activity is needed for the Muv phenotype. Temperature-shift experiments show that *glp-1(q35)* acts before the first P(3–8).p division (Fig. 3c), at the time of VPC determination¹². At this time most *glp-1(+)* messenger RNA is found in the soma¹; *lin-12(d)* activity is also required at this stage to determine VH2 fate⁶.

The parallels between the effects of *glp-1(q35)* and *lin-12(d)* on vulval fate are striking. Both promote the VH2 fate among the VPCs, independently of an AC or VH1 cell; both result from a gain of gene function acting at the time of VPC determination. These similarities suggested that *glp-1(q35)* might affect VPC development by activating *lin-12* itself. To test this hypothesis we examined whether functional *lin-12* was necessary for the *glp-1(q35)* Muv phenotype. We found that *smg-1;lin-12(0)glp-1(q35)* animals still form pseudovulvae, either with a gonad or without one (Fig. 1e,f and 2b, rows 18–26). Thus, *lin-12* activity is not required for the *glp-1(q35)* Muv phenotype. Moreover, this is the first report of a VPC able to adopt a VH2 fate in the absence of functional *lin-12*. We therefore propose that *glp-1(q35)* can substitute for *lin-12* to direct development of the vulval hypodermis.

Although the effect of *glp-1(q35)* on the VPCs mimics that of *lin-12(d)*, other fate transformations typical of *lin-12(d)* do not appear to be affected by *glp-1(q35)* (data not shown). We do not know whether this difference reflects a dissimilarity in gene expression or protein function. For example, *glp-1(q35)* may not be expressed in the same tissues as *lin-12(d)*, and therefore could not alter these other cell fate decisions.

The *glp-1(q35)* mutation is associated with a C to T base transition that changes the triplet encoding arginine at position

1174 to a stop codon, truncating the predicted *glp-1* peptide by 122 amino acids (Fig. 4). Both the recessive Glp and dominant Muv phenotypes are likely to stem from this single lesion as both are affected by *smg-1* and both are temperature-sensitive. Furthermore, because *lin-12* activity is not required, the Muv phenotype associated with *glp-1(q35)* cannot be explained by a closely linked *lin-12(d)* mutation.

The region removed by the *glp-1(q35)* mutation is unlike that of *lin-12*, except for a short PEST sequence (Fig. 4b). Such sequences are thought to be degradation signals¹⁴. Supporting this hypothesis, mutations that alter a PEST-like sequence in the *ftz* gene stabilize the protein and cause dominant, gain-of-function phenotypes¹⁵. Similarly, the *glp-1(q35)* Muv phenotype may result from an increase of *glp-1* protein. In addition, the carboxy-terminal truncation may either destroy a more specific negative regulatory domain, thereby derepressing normal *glp-1* activity, or alter the specificity such that the *glp-1(q35)* protein can interact with factors that the normal *glp-1* product cannot.

The *glp-1(q35)* mutation generates loss-of-function Glp phenotypes in addition to the gain-of-function Muv phenotype. This reduction in *glp-1* activity may reflect a less active protein, or a decreased amount of protein resulting from destabilized mRNA, an effect seen with other nonsense mutations (see for example, ref 16). Consistent with the latter hypothesis is the efficient suppression of the Glp phenotypes by *smg-1*. Because the *glp-1(q35)* truncated protein is functional in a *smg-1* background we propose that the carboxy-terminal 122 amino acids of the *glp-1* protein are not essential. Furthermore, we suggest that the distinct roles of *glp-1* and *lin-12* in development rely on gene-specific regulation rather than on unique functional domains.

As wild-type *glp-1* acts in the germ line to promote mitosis³, we speculate that *glp-1(q35)* may act in the vulval hypodermis by promoting proliferation. Likewise, some cell fate decisions

that require *lin-12* are also proliferative⁶. Many mammalian growth factors (for example, colony-stimulating factor-1; ref. 17) promote both proliferation and differentiation. Perhaps these processes are similarly linked in *C. elegans*, and by driving proliferation, *glp-1(q35)* influences a VPCs subsequent fate. □

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Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs

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DUCHENNE'S muscular dystrophy (DMD), which affects one in 3,500 males, causes progressive myopathy of skeletal and cardiac muscles and premature death¹. One approach to treatment would be to introduce the normal dystrophin gene into diseased muscle cells. When pure plasmid DNA is injected into rodent skeletal² or cardiac muscle³⁻⁵, the cells express reporter genes. We now show that a 12-kilobase full-length human dystrophin complementary DNA gene and a 6.3-kilobase Becker-like gene⁶ can be expressed in cultured cells and *in vivo*. When the human dystrophin expression plasmids are injected intramuscularly into dystrophin-deficient mdx mice, the human dystrophin proteins are present in the cytoplasm and sarcolemma of ~1% of the myofibres. Myofibres expressing human dystrophin contain an increased proportion of peripheral nuclei. The results indicate that transfer of the dystrophin gene into the myofibres of DMD patients could

be beneficial, but a larger number of genetically modified myofibres will be necessary for clinical efficacy.

A human full-length⁷ or a Becker-like cDNA dystrophin sequence was inserted into expression vectors containing either the Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoters (Fig. 1). In cultured Cos cells transfected with constructs containing either the Becker-like sequence (pCMVDy-B) or the construct containing the full-length dystrophin sequence (pRSVDy), a dystrophin protein of relative molecular mass (M_r) 200,000 (200K) (Fig. 2a, lane 3) or 427K (Fig. 2a, lane 4), respectively, was detected on immunoblots. Some 30% of Cos

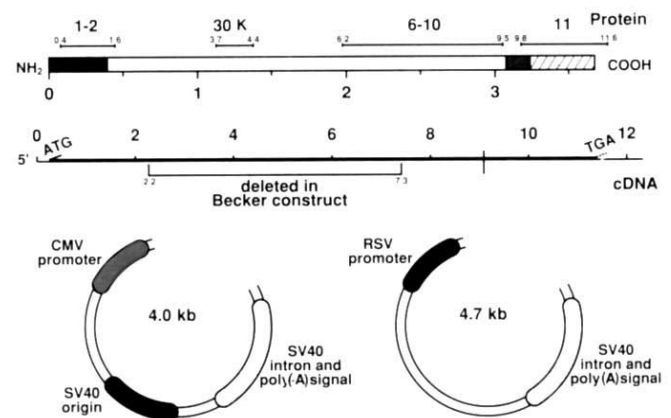


FIG. 1 Diagrammatic representation of the human dystrophin cDNAs, protein and related antibodies. The full-length dystrophin construct corresponds to a segment of cDNA of ~12 kb (112 bp to 12,101 bp; ref. 25) encoding the principal skeletal muscle isoform of human dystrophin⁷. The 6.3-kb Becker-like dystrophin construct was made in three parts. (1) PCR amplification across the exon 16/49 boundary: plasmid pCf56a and the 2.2-kb *EcoRI* insert of pCf27 (ref. 26) were amplified by PCR using oligonucleotides M13-forward (BRL) plus 5'-GAAAAGAGTACAGCACAGAACTGAAATAGCAGTT-3' (324J-LF) and 5'-CTGGCCGTTTAAAGCG-3' (324J-F) plus 5'-AACTGCTATTTTCAGTTTCTGTGCTGACTCTTTTC-3' (324J-LR), respectively. Oligonucleotides 324J-LF and 324J-LR are complementary with the dystrophin exon 16/49 boundary at the mid-position in each oligonucleotide. Amplification by PCR consisted of 15 cycles of 94 °C for 1 min, 45 °C for 30 s and 72 °C for 1 min with each oligonucleotide present at 1 μ M²⁷. One-fiftieth of each PCR was pooled and amplified for 30 cycles using oligonucleotides M13-forward and 324J-F (5' nucleotide at dystrophin position 2,015). The DNA product was subsequently digested with *XbaI* plus *EcoRI* and the 740-bp fragment introduced into double-digested pUC18 (Stratagene). A recombinant plasmid containing the correct PCR product (5' and 3' ends at dystrophin nucleotide positions 2,036 and 7,879) was determined by restriction enzyme digestion and sequencing of both DNA strands using the Sequenase Kit (USB). (2) 5'-end construction: the plasmid recombinant and pCf27 were digested with *EcoRI* then with *BglII* to give complete or partial digestion, respectively, at dystrophin nucleotide position 2,083. Fragments of ~690 bp (boundary PCR clone) and 1,971 bp (Cf27 from nucleotide position 112 to 2,083) were pooled, ligated in equimolar amounts and digested with *EcoRI*. The 2.7-kb fragment was isolated and ligated into the *EcoRI* site of pUC18. (3) 3'-end construction: the 4.3-kb *EcoRI* insert of pCf115 (ref. 26) was partially digested with *HindIII* (at nucleotide position 11,512), and the 3.6-kb dystrophin fragment ligated into *EcoRI* plus *SmaI*-digested pUC18. This recombinant plasmid was digested with *EcoRI* (nucleotide position 7,876) plus *SalI* (3' end in multiple cloning site (MCS) of pUC18) and the dystrophin insert ligated with an equimolar amount of the 2.7-kb insert recovered from the recombinant, which had been digested to completion with *SalI* (5' end in MCS of pUC18) and partially with *EcoRI* (nucleotide position 7,875). The ligated DNAs were digested with *SalI* and the 6.3-kb fragment ligated into the *SalI* site of pUC18. The dystrophin cDNAs were inserted into the RSV promoter construct derived from pRSVLacZ (ref. 24) or the polylinker site of the pCDNA1 plasmid (Invitrogen). pCDNA1 contains a CMV promoter and the SV40 poly(A) addition signal, intron and origin of replication. The regions of dystrophin that were used to produce antibodies are represented by the bars above the protein⁹⁻¹⁴. The numbers at the beginning and end of the brackets indicate the corresponding dystrophin cDNA sequences.

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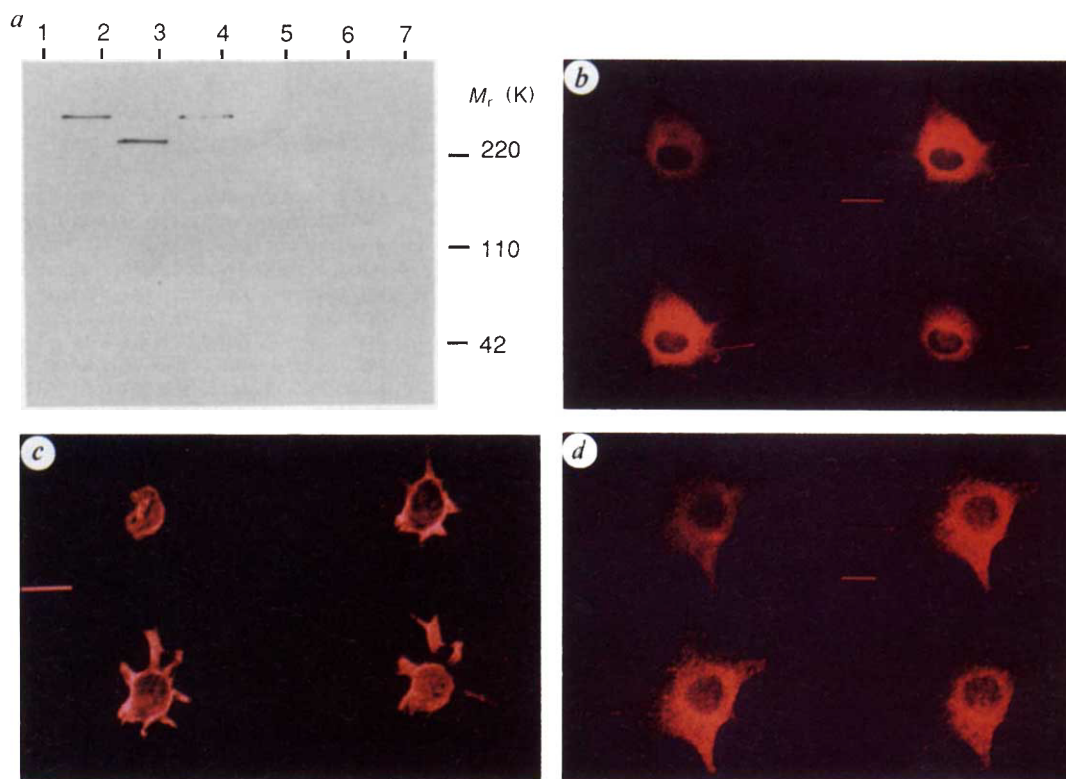
cells transfected with pCMVDy-B (Fig. 2*b*) and about 5% of Cos cells transfected with pRSVDy (Fig. 2*d*) contained dystrophin protein significantly increased above the background levels staining in control Cos cells. Mouse dystrophin has been localized to the cell membrane in Cos cells transfected with a cDNA construct⁸. Most (~80%) of our dystrophin-positive cells contain dystrophin in the cytoplasm (Fig. 2*b* and *d*). A few of the pCMVDy-B or pRSVDy-transfected cells that showed dystrophin immunoreactivity at the cell perimeter (Fig. 2*c*) were anucleated, rounder, less attached, and not viable as judged by the trypan blue-exclusion test.

When these dystrophin expression plasmids were injected intramuscularly into dystrophin-deficient mdx mice, human dystrophin was detected (Table 1; Fig. 3). Seven days after injection of 400 µg of either pRSVDy or pRSVDy-B into the rectus femoris of the quadriceps of four- to six-week-old mdx mice, the muscles were excised and stained for dystrophin immunoreactivity using several antibodies (Fig. 1). Muscles in mdx mice contain naturally occurring myofibres with dystrophin immunoreactivity which have been described as 'revertants'⁹. The presence of these 'revertants' necessitated a comparison with a control muscle, so we used the contralateral quadriceps

injected with the plasmid pRSVCAT which expresses chloramphenicol acetyl transferase¹⁰. In the muscles injected with either pRSVDy or pRSVDy-B, most of the cells with dystrophin immunoreactivity were found about 5 mm proximal to the injection site, where the injection fluid dispersed, although there were many dystrophin-positive cells throughout the quadriceps muscle (Table 1). Using the most sensitive anti-dystrophin antibody, 6-10, a mean of 103 (s.d. = 52) dystrophin-positive fibres were present in the proximal part of pRSVDy-B-injected muscle, as compared with a mean of 58 (s.d. = 26) dystrophin-positive fibres in the proximal part of control pRSVCAT-injected muscle (Wilcoxon rank sum test, $p < 0.001$) (Table 1; Fig. 3*b* and *c*). Also, a significantly greater number of dystrophin-positive fibres ($P < 0.02$) were observed in pRSVDy-injected muscles (mean, 74; s.d. = 12) than in the control pRSVCAT-injected muscles (mean, 47; s.d. = 12) (Table 1*b*; Fig. 3*d*). These results indicate that ~1% of the ~4,000 rectus femoris myofibres expressed human dystrophin.

There were other differences in staining between control muscles and muscles injected with pRSVDy or pRSVDy-B that were further evidence of *in vivo* expression, besides the increased number of stained fibres. Whereas the same myofibres in

FIG. 2 Expression of human dystrophin in cultured cells. *a*, Immunoblot; lane 1, control Cos cells transfected with pRSVCAT; lane 2, normal mouse muscle; lane 3, Cos cells transfected with pCMVDy-B; lane 4, Cos cells transfected with pRSVDy; lane 5, mdx mouse muscle; lane 6, extracts from lanes 3 and 5 mixed 1:1; lane 7, extracts from lanes 3 and 5 mixed 1:4. Migration of M_r markers is indicated on the right. *b-d*, Confocal fluorescent images of transfected Cos cells immunohistochemically stained for human dystrophin (scale bars, 10 µm). *b*, One pCMVDy-B-transfected Cos cell shown at 4 different levels; *c*, peripherally stained Cos cell from a pCMVDy-B-transfected culture shown at 4 different levels; *d*, one pRSVDy-transfected Cos cell shown at 4 different levels. Similar results on immunoblots and immunohistochemistry were obtained with the 1-2 and 11 dystrophin antibodies.



with 6-10 primary antibody (1:2,000 dilution) in TBST containing 0.2% BSA overnight. After washing the membrane three times with TBST, it was incubated with horseradish peroxidase-conjugated anti-rabbit IgG in TBST containing 0.2% BSA for 2 h. The colour reaction was developed in 0.06% 4-chloro-1-naphthol (Biorad), 20% (v/v) methanol and 0.2% hydrogen peroxide in Tris-buffered saline for 10 min. Cells to be studied immunohistochemically were fixed in acetone for 1 min, dried, then preblocked with 2% horse serum (Vector Labs) in phosphate-buffered saline, pH 7.4, and 0.1% Triton-X (PBST) for 2 h. The 6-10 primary antibody diluted 1:2,000 in PBST containing 1% horse serum was applied overnight at 4°C. The biotinylated secondary antibody against rabbit IgG (Amersham) was applied for 1-2 h and an avidin-Texas Red conjugate (BRL) was then applied for 30 min. Between each step, cells were washed three times with PBS for 5 min. Finally, the coverslips were mounted on glass slides with Gel/Mount (Bio-medex). Fluorescent images were obtained with a Nikon photomicroscope fitted with laser confocal imaging system (Biorad MRC-600).

METHODS. Three million Cos cells in 100-mm tissue culture plates were transfected with complexes of either 15 µg pCMVDy-B with 45 µg lipofectin (BRL) or of 50 µg pRSVDy DNA with 150 µg lipofectin as described²⁸. Two days after transfection cells were either stained immunohistochemically or lysed for immunoblot analysis. Lysis of cells was in 200 µl 10 mM Tris, pH 8.0, 10% SDS, 1 mM DTT with protease-inhibitor cocktail (1 mM PMSF, 1 mM EDTA, 1 mM benzamide, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ pepstatin, 1 µg ml⁻¹ aprotinin). The muscle tissues were homogenized in the same buffer using 20 × v/w. Immunoblotting was by a modification of a previously published procedure²⁹. Briefly, about 10 µg of protein extracts were separated on an 8% polyacrylamide (Biorad) gel in a Hoefer SE 250 Mighty Small electrophoresis unit. The gel was transferred to an Immobolin-P membrane (Millipore) in a Hoefer Mighty Small Transphor: TE 22 apparatus using 400 mA (constant current) for one hour at 4°C and a transfer buffer²⁹ without mercaptoethanol. After the transfer, the membrane was pretreated with TBST (50 mM Tris, 0.9% NaCl, pH 7.4, 0.05% Tween 20 (Biorad) containing 1% bovine serum albumin (BSA) for 2 h. Then the membrane was incubated

pRSVDy-injected muscle were immunoreactive on serial sections with the 1-2, 11, 30K and 6-10 antibodies¹¹⁻¹⁴, myofibres in pRSVDy-B-injected muscle were not immunoreactive against the 30K antibody because the 30K epitopes are not present in the Becker-like construct (Fig. 1). Dystrophin immunoreactivity was primarily located at the cell surface in the fibres of both the experimental and control muscles. But only the pRSVDy-B or pRSVDy-injected muscles contained myofibres with both sarcolemmal and a fainter cytoplasmic dystrophin immunoreactivity (Table 1; Fig. 3c and d) which may result from dystrophin overexpression. The predominantly sarcolemmal localization suggests that the recombinant human dystrophins can complex with the membrane glycoproteins^{15,16}.

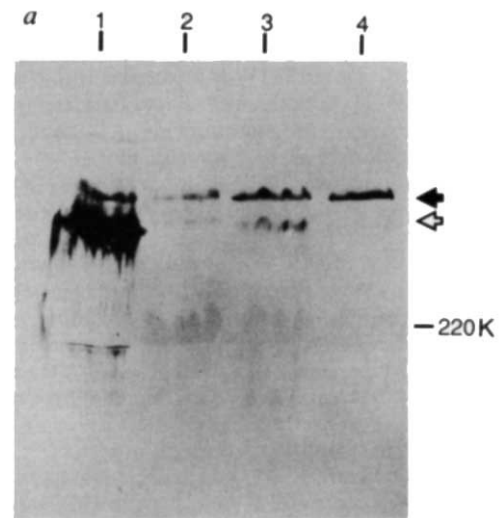
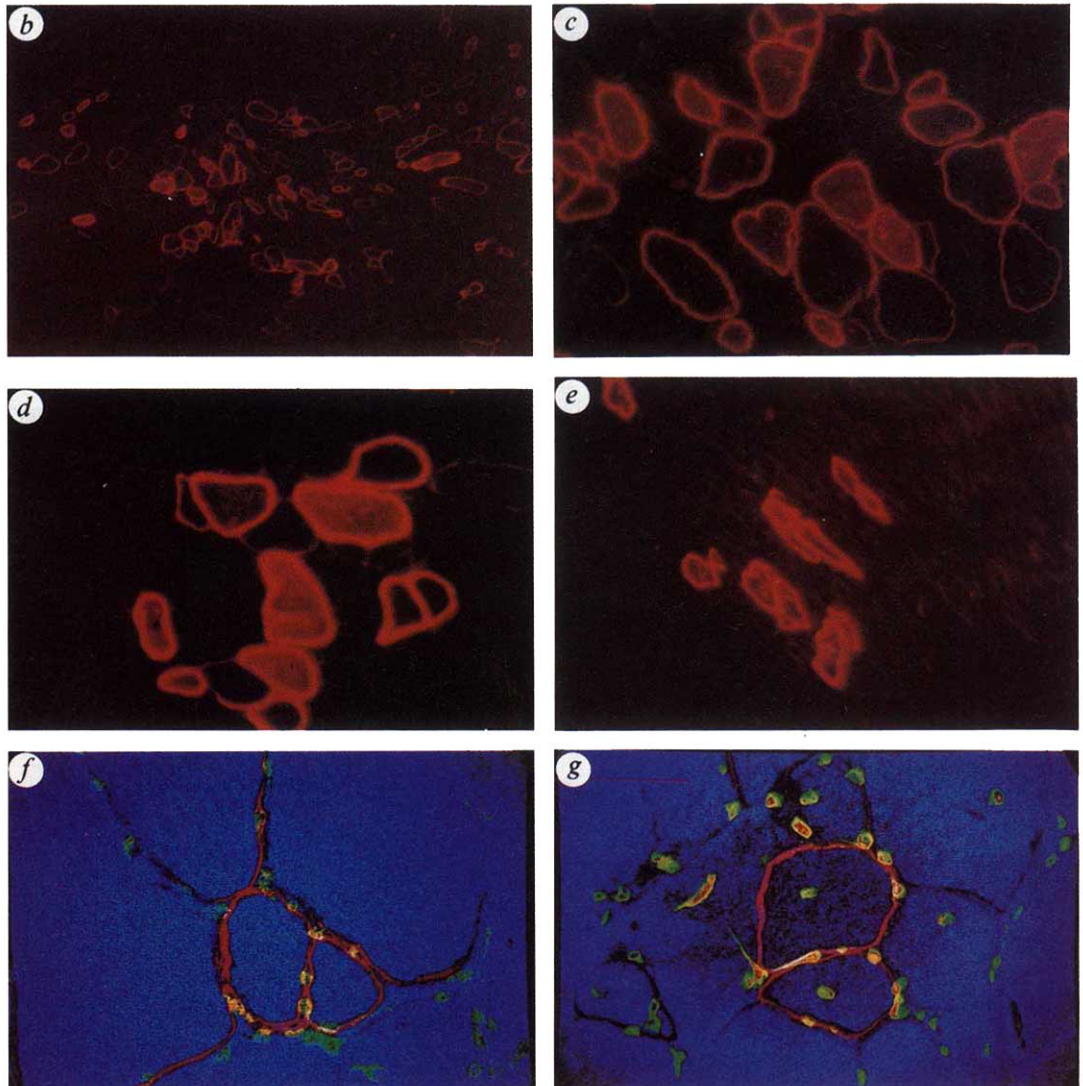


FIG. 3 Expression of human dystrophin in muscles of mdx mice. *a*, Immunoblot: lane 1, uninjected, normal mouse muscle; lanes 2 and 3, pRSVDy injected mdx muscle; lane 4, pRSVCAT-injected mdx muscle. Open arrow indicates the location of full-length dystrophin. Closed arrow indicates the location of nebulin which was identified on a different immunoblot by the use of anti-nebulin antibodies (Sigma). *b-g*, Immunohistochemical staining of injected quadriceps. *b*, pRSVDy-B injected (6 $\times$); *c*, pRSVDy-B injected (25 $\times$); *d*, pRSVDy injected (25 $\times$); *e*, heart injected with pCMVDy-B (16 $\times$); *f*, confocal fluorescent image of pRSVDy-injected muscle co-stained with 1 $\mu\text{g ml}^{-1}$ acridine orange (Eastman Kodak); *g*, confocal fluorescent image of pRSVCAT-injected muscle co-stained with acridine orange; scale bar, 50 μm .

METHODS. 4-6-week-old mdx mice (Jackson Labs) were injected with 400 μg dystrophin constructs into one side of the quadriceps muscle and with pRSVCAT construct¹⁰ in the other quadriceps muscle in physiological saline^{2,30}. Immunoblotting was as described in the legend to Fig. 2, except that 40 μg muscle protein, biotinylated anti-rabbit IgG (Amersham) and avidin horseradish peroxidase conjugate (Vector) were used. In injected muscles, trimmed sections containing dystrophin-positive cells as determined by immunohistochemistry were analysed. For immunohistochemistry, the entire quadriceps was placed on cork and frozen in liquid N₂-cooled isopentane. Serial sections (6- μm) were fixed in acetone and stained for dystrophin as described for Fig. 2. Either a Leitz epifluorescence microscope or a Nikon photomicroscope fitted with laser confocal imaging system (Biorad MRC-600) was used to obtain the photomicrographs.



Evidence for *in vivo* expression has also been obtained in studies on the myocardium, in which 'revertants' in the hearts of ten mdx mice were found only in isolated groups of one to three cells: on average, the number of revertants was comparable to that in a previous study⁹, apart from one cluster of eight cardiac revertants. In pRSVDy-B-injected cardiac tissue, we observed groups of up to thirteen cardiac cells with sarcolemmal and cytoplasmic dystrophin staining (Fig. 3e), confirming that there was expression *in vivo*. To verify that it was full-sized human dystrophin that was being expressed *in vivo* in pRSVDy-injected muscle we showed that a 427K dystrophin protein was present on immunoblots (Fig. 3a, lanes 2 and 3); pRSVCAT-injected muscle contained a much fainter 427K band (lane 4). The 6-10 antibody's cross-reaction with the larger nebulin protein served as an internal control for the amount of protein loaded and transferred. Large amounts of 220K myosin obscured detection of the 200K Becker-like protein in pRSVDy-B injected muscle (Fig. 2a, lanes 6 and 7). We also have preliminary results from the reverse transcriptase polymerase chain reaction (PCR)¹⁷ that further confirm expression *in vivo*.

Dystrophin deficiency in the mdx muscle results in a variety of effects, including centralized nuclei¹⁸⁻²³. In mdx muscle injected with pRSVDy-B or pRSVDy, 40% to 50% of the dystrophin-positive fibres ($n=50$) contained peripheral nuclei, compared with ~20% in dystrophin-negative cells in all muscles ($n=50$) or in dystrophin-positive cells of control pRSVCAT-injected muscles ($n=50$) ($\chi^2=22$, $P<0.001$) (Fig. 3f and g). After the injection of pRSVLac-Z (ref. 24) into mdx muscle, 83% of β -galactosidase-positive myofibres had central nuclei ($n=42$), indicating that myofibres with peripheral nuclei do not express injected plasmids any more readily. Why 'revertant' myofibres with dystrophin immunoreactivity do not have increased numbers of peripheral nuclei is unclear.

The ability of transduced human dystrophin expression to correct an effect of dystrophin deficiency in mice after birth

suggests that the delivery and expression of exogenous dystrophin genes could correct other effects of dystrophin deficiency, such as increased intracellular calcium. Although gene transfer may offer some advantages over myoblast transfer²¹⁻²³, the efficiency of this gene transfer technique needs to be increased before it can be used clinically. □

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TABLE 1 The number of dystrophin-positive myofibres in injected mdx muscle

Injection of Becker-like construct

Muscle number	Location	Mean number of dystrophin-positive fibres*			
		pRSVDy-B	pRSVCAT	pRSVDy-B	pRSVCAT
1	Proximal	28	18	1	0
	Central	34	28	3	0
2	Proximal	135	75	10	0
	Central	114	59	7	0
3	Proximal	195	102	15	0
	Central	110	73	5	0
4	Proximal	114	68	11	0
	Central	80	56	8	0
5	Proximal	86	47	8	0
	Central	71	42	5	0
6	Proximal	124	61	10	0
	Central	87	49	6	0
7	Proximal	54	36	2	0
	Central	55	56	0	0
8	Proximal	85	53	6	0

Injection of full-length construct

		pRSVDy	pRSVCAT	pRSVDy	pRSVCAT
1	Proximal	89	50	7	0
	Central	64	44	2	0
2	Proximal	71	44	6	0
	Central	52	39	0	0
3	Proximal	69	57	4	0
	Central	48	46	7	0
4	Proximal	81	55	3	0
	Central	65	44	5	0
5	Proximal	58	28	8	0

*For each part of each muscle, the number of fibres in three adjacent sections were counted and averaged (s.d. < 5).

Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA

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SPLICING of pre-messenger RNA in eukaryotic cells occurs in a multicomponent complex termed the spliceosome, which contains small nuclear ribonucleoprotein particles (snRNPs), protein factors and substrate pre-mRNA¹⁻³. Assembly of the spliceosome involves the stepwise binding of snRNPs and protein factors to the pre-mRNA through a poorly understood mechanism which probably involves specific RNA-RNA, RNA-protein and protein-protein interactions. Of particular interest are the interactions between snRNPs, which are likely to be important not only for assembly of the spliceosome but also for catalysis. U1 snRNP interacts with the 5' splice site and U2 snRNP with the branch site of the pre-mRNA; both of these interactions involve Watson-Crick base pairing⁴⁻⁹. But very little is known about how other factors such as the U4/U6 and U5 snRNPs reach the spliceosome and function in splicing. Here we report evidence that U6 snRNA interacts directly with U2 snRNA by a mechanism involving base-pairing, and that this interaction can be necessary for splicing of a mammalian pre-mRNA *in vivo*.

Simian virus 40 (SV40) early pre-mRNA is alternatively spliced to generate large-T and small-t mRNAs by utilizing different 5' splice sites and a shared 3' splice site. Large-T splicing uses multiple branch acceptors whereas small-t splicing, constrained by its small intron size (66 nucleotides), can use only one, an adenosine at -18 relative to the 3' splice site¹⁰⁻¹³. We previously used a cotransfection assay with this system to show that the recognition of the poorly conserved mammalian pre-mRNA branch site sequence by U2 snRNP involves base pairing⁷. An SV40 mutant containing a 3-base pair (bp) substitution in the branch site region (SV-AGU) which was defective for small-t splicing could be efficiently suppressed by an appropriately mutated U2 snRNA (U2-UCA). We have now exploited this situation to prove the existence of a base-pairing interaction between U2 and U6 snRNAs.

Earlier studies suggested that the 5' terminal region (nucleotides 1-15) of U2 RNA is required for spliceosome assembly and efficient splicing *in vitro*^{14,15}. To test whether specific bases in this region might be important for splicing *in vivo*, eight single base substitutions, at positions 2, 3, 5 and 7-10, were constructed in U2-UCA, as illustrated in Fig. 1. Each mutant plasmid was cotransfected into human 293 cells together with SV-AGU, and total cytoplasmic RNA was analysed by S1 nuclease digestion to determine the effects of these mutations on the ability of U2-UCA to restore small-t splicing (Fig. 2). As before⁷, the ratio of small-t to large-T mRNA produced from 293 cells transfected with SV-AGU and U2-UCA was close to that from cells transfected with the SV40 wild-type plasmid (SV-WT). The eight mutants affected splicing in several ways. Small-t splicing was virtually undetectable with mutants U2-3G, U2-5G, U2-7G and U2-8A, was moderately reduced with U2-2G and U2-10A, but was barely affected with mutant U2-9G. The observed inactivation of small-t splicing was not likely to be due to defects in

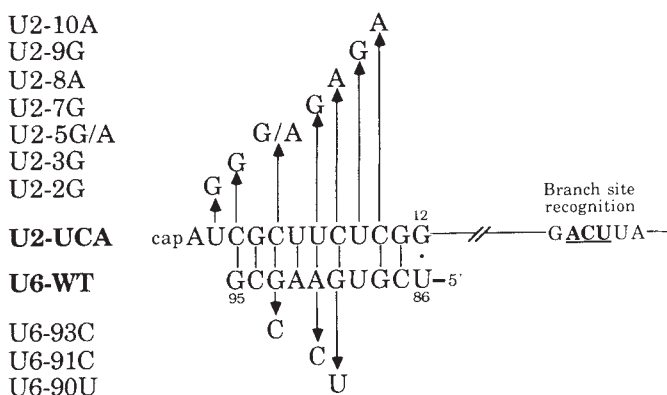


FIG. 1 Diagram of the base-pairing interaction between U2 and U6 snRNAs and of the mutations analysed here. The sequence of the U2 snRNA 5' terminal region and a complementary sequence in U6 snRNA (U6-WT) are indicated. Numbers indicate the distance downstream of the transcription start sites in each molecule. The base changes introduced in these sequences are shown, and the resulting mutant plasmids were named to indicate these changes. The region of U2 snRNA involved in base pairing with the pre-mRNA branch site is also indicated; the bases mutated in U2-UCA are in bold type.

METHODS. U2 sequences were excised from the U2-UCA plasmid⁷ by digesting with *EcoRI* and *PstI* (the *PstI* site was blunt-ended by treatment with T4 DNA polymerase). This fragment was inserted into pBS-SK⁺ between the *EcoRI* and filled-in *NotI* sites. The single-stranded template for oligonucleotide-directed mutagenesis was prepared by superinfection with the helper phage M13K07 (BioRad). Oligonucleotide-directed mutagenesis was essentially as described by Kunkel *et al.*³⁰, and mutants were identified by DNA sequencing³¹. U2-WT and U2-UCA were previously constructed in pGEM4 (ref. 7). U6 DNA was excised from pGEM/U6 (ref. 17) by digesting with *HindIII* and *EcoRI* and inserted into pBS-SK⁺. This construct contains ~470 base pairs (bp) of 5' flanking sequence, 106 bp of coding sequence, and ~220 bp of 3' flanking sequence. Mutagenesis was carried out as described above.

expression or stability of the mutant U2 snRNAs, because analysis of several mutants in this region, using a primer extension assay with the appropriate dideoxynucleotide to distinguish transfected mutant from endogenous wild-type U2 snRNA, revealed that they all accumulated to high levels in the nuclei of transfected cells (results not shown).

One explanation for our results is that the 5' end of U2 snRNA must at some stage during splicing base pair with another snRNA, and the defective mutants are unable to do so. Indeed, two base-pairing interactions with different regions of U6 snRNA are possible: U2 nucleotides 5-10 with U6 nucleotides 44-49, or U2 nucleotides 3-12 with U6 nucleotides 86-95. The latter interaction is particularly appealing for two reasons. First, the behaviour of all eight mutants analysed is consistent with this interaction (Fig. 1). Specifically, U2 positions 2 and 9 are not predicted to be base-paired, and mutations at these positions had relatively small effects on U2 function. Position 10, which had an intermediate effect, is downstream of the six consecutive base pairs extending from position 3 to 8, and thus might not be critical. All other mutations that greatly reduce splicing also alter bases in a 6-nucleotide stretch which would form the core

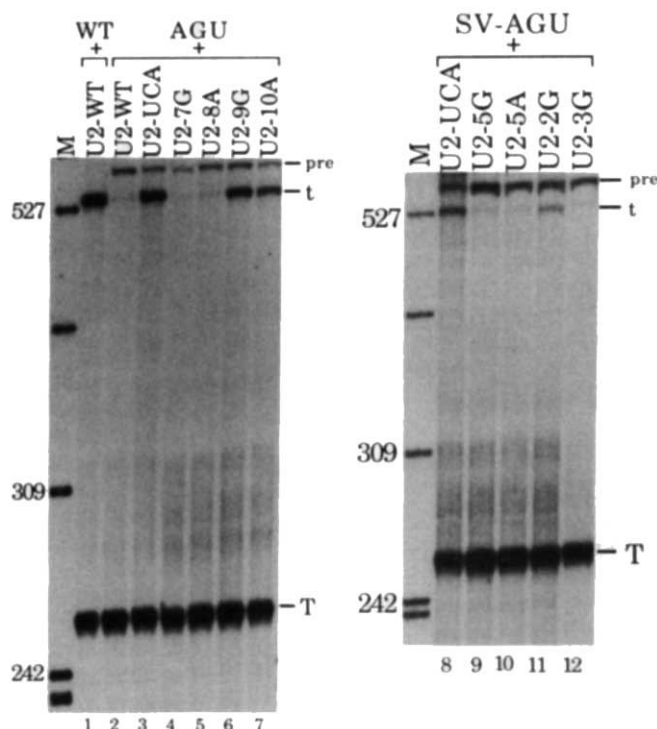


FIG. 2 Effects of mutations in U2 snRNA on SV40 small-t splicing *in vivo*. Cytoplasmic RNA was extracted from 293 cells cotransfected with a plasmid containing the wild-type SV40 early region (SV-WT) or a mutant containing a 3-bp substitution in the branch site (SV-AGU), plus either the wild-type U2 gene, the U2 suppressor U2-UCA, or U2-UCA with the indicated additional mutations in the 5' terminal region. RNA was analysed by nuclease S1 analysis. T, Large T mRNA; t, small t mRNA; pre, unspliced precursor; M, DNA markers (sizes indicated in nucleotides).

METHODS. Human 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections were performed as described⁷, except that 10 µg SV40 plasmids (SV-WT or SV-AGU) plus 40 µg of the indicated U2 plasmid was used in each sample. Transient expression was terminated 48 h after DNA addition and cytoplasmic RNA was isolated as before⁷. The left and right panels are derived from separate transfections. Nuclease S1 analysis⁷ used a 3'-end labelled *HindIII*-*BsmI* DNA probe (nucleotides 5,171-4,528) isolated from SV-WT (pSTER; see ref. 12). The use of this probe permitted resolution of unspliced pre-mRNA and undigested probe when SV-AGU, but not SV-WT, was used in transfections. Nuclease S1-resistant products were resolved by electrophoresis through 5% polyacrylamide-7 M urea gels, which were dried and exposed to X-ray film at -70 °C in the presence of intensifying screens.

of this base-pairing interaction. Second, a fraction of U2 and U6 snRNAs in nuclear extracts can be chemically crosslinked, and this crosslinking involves bases upstream of position 15 in U2 snRNA and downstream of position 85 in U6 snRNA¹⁶. As noted already¹⁶, this is consistent with the latter base-pairing interaction.

To test whether disruption of base pairing between U2 and U6 snRNAs was responsible for the inactivated splicing function of the U2 mutants analysed above, we constructed three compensatory mutations in the 3' terminal region of a cloned human U6 snRNA gene¹⁷ (see Fig. 1). Cells (293) were then cotransfected with SV-AGU plus each U2 mutant and either wild-type U6, the potential suppressor, or a noncompensating U6 mutant. The results of nuclease S1 analysis of RNA extracted from these cells is shown in Fig. 3. Strikingly, the U6 mutant U6-91C resulted in a significant increase in small-t splicing when cotransfected with the complementary U2 mutant, U2-7G (Fig. 3a, lane 4). U6-91C did not affect small-t or large-T splicing when cotransfected with either U2-UCA or U2-8A (lanes 2 and 5), indicating that the effect of U2-7G on small-t splicing was allele-specific. Likewise, the activity of U2-7G was not restored by cotransfection with U6 wild type (lane 3), indicating that this mutation could not be suppressed by a nonspecific effect, reflecting overexpression of U6 snRNA. Small-t splicing was also rescued by U6-93C when this plasmid was cotransfected with U2-5G (lane 8). U6-93C did not restore small-t splicing when cotransfected with either U2-5A or U2-3G (lanes 9 and 10), establishing the strict allele specificity of the suppression, and the activity of U2-5G was not affected by cotransfection

with U6 wild type (lane 7), again indicating the specificity of the interaction. Small-t splicing was restored, although at a somewhat lower efficiency, when U2-8A was cotransfected with U6-90U (Fig. 3b, lane 4). As with U6-91C and U6-93C, the restoration of small-t splicing was allele-specific: small-t splicing was not affected when U6-90U was cotransfected with U2-UCA or U2-7G (lanes 2 and 5). The splicing activity of U2-8A also was not restored after cotransfection with U6 wild type (lane 3).

Our results establish that base-pairing between U2 and U6 snRNAs is required for efficient splicing of a mammalian pre-mRNA *in vivo*. A similar conclusion was drawn from experiments employing a human globin pre-mRNA³², ruling out the possibility that our findings were specific for the SV40 early transcript. We note that our assay, involving *cis* competition between large-T and small-t 5' splice sites, is potentially very sensitive, and we thus cannot say whether U2-U6 base pairing is absolutely required for splicing, or whether it functions to enhance the rate or efficiency of the reaction. Perhaps consistent with this last possibility, functional analysis of yeast U2 and U6 snRNAs indicates that deletion of nine nucleotides in U6 that would be involved in U2-U6 base pairing causes only a cold-sensitive growth phenotype¹⁸, and mutations in several of the critical U2 nucleotides has no detectable effect on growth (D. Field and J. Friesen, personal communication). Also, splicing in a yeast *in vitro* system is only moderately affected by 3' truncations of U6 snRNA¹⁹. These results could reflect either an important difference in splicing between metazoans and yeast, or the relative sensitivities of the assays. For example, in yeast

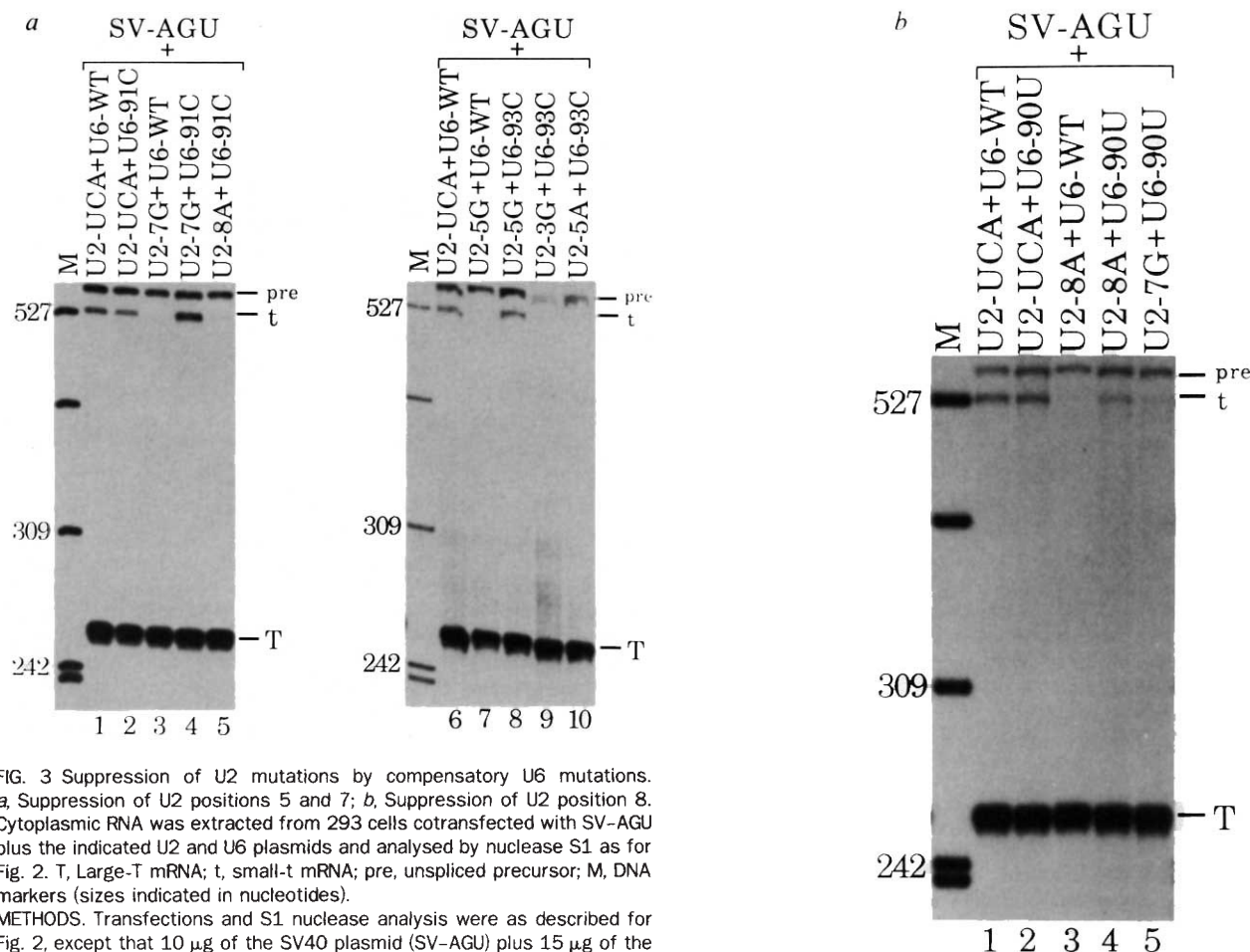


FIG. 3 Suppression of U2 mutations by compensatory U6 mutations. *a*, Suppression of U2 positions 5 and 7; *b*, Suppression of U2 position 8. Cytoplasmic RNA was extracted from 293 cells cotransfected with SV-AGU plus the indicated U2 and U6 plasmids and analysed by nuclease S1 as for Fig. 2. T, Large-T mRNA; t, small-t mRNA; pre, unspliced precursor; M, DNA markers (sizes indicated in nucleotides).

METHODS. Transfections and S1 nuclease analysis were as described for Fig. 2, except that 10 μ g of the SV40 plasmid (SV-AGU) plus 15 μ g of the indicated U2 plasmid and 25 μ g of the indicated U6 plasmid were used in each sample. Preliminary primer extension experiments using a U6 gene mutated at nucleotides 44–47 indicated that U6 snRNA was efficiently

expressed from the transfected DNA (results not shown). Three panels in *a* (left and right) and *b* are derived from separate transfections.

U1 snRNA levels can be reduced by more than a factor of 10 without affecting growth rate²⁰.

The base-pairing interaction we have demonstrated is, however, consistent with previous studies on the function of U2 and U6 RNAs in metazoan systems. Oligonucleotide-directed degradation experiments have shown that disruption of nucleotides 1–15 of U2 RNA or nucleotides 78–95 of U6 RNA abolishes or greatly reduces pre-mRNA splicing *in vitro*^{14,21}. Also, a complementation assay in *X. laevis* oocytes has indicated that deletion of nucleotides 77–89 or 90–98 prevents U6 snRNA from activating splicing in oocytes in which endogenous U6 snRNA is inactivated²². Also, when distinct domains of snRNAs are masked with antisense 2'-methylated RNA oligonucleotides, then nucleotides 1–20 of U2 RNA and nucleotides 82–101 of U6 RNA are necessary for splicing in HeLa nuclear extracts^{23,24}. Analysis of spliceosome assembly using 2'-methylated oligonucleotides has shown that masking of nucleotides 1–20 of U2 RNA blocks the formation of the complete spliceosome, but not of the prespliceosome, which contains only U1 and U2 snRNPs²³; this suggests that the 5' domain of U2 RNA interacts with other splicing components after U2 snRNP binding to the pre-mRNA. Another *in vitro* analysis of U6 RNA function has indicated that deletions or substitutions encompassing positions 90 to 95 in U6 RNA prevent spliceosome assembly and/or splicing (A. Bindereif, personal communication). Taken together, these results are all consistent with the notion that the U2–U6 base-pairing interaction is critical for efficient splicing.

Phylogenetic analysis supports the existence of a stem-loop structure in U2 snRNA, encompassing nucleotides 7 to 14 and 19 to 26 (ref. 25): this region overlaps extensively with the region of U2 that we have shown is involved in base pairing with U6, so it is possible that U2 snRNA undergoes a conformational change at some point during the splicing reaction. Perhaps the intramolecular stem-loop is required during snRNP assembly and/or for the initial interaction of U2 with the pre-mRNA. Melting of at least part of this stem would then allow base pairing with U6, possibly for a subsequent splicing step.

The U2–U6 interaction is the fourth example of RNA–RNA base pairing involved in spliceosome assembly and/or splicing, the others being the U1–5' splice site^{4–6}, U2-branch site^{7–9}, and U4–U6 (refs 22, 26) interactions. The U2–U6 base pairing, however, is the first involving two distinct snRNPs. Figure 4 shows the two regions of U2 snRNA that participate in intermolecular base pairing. Specifically, nucleotides 33–38 base pair with the pre-mRNA branch site, and nucleotides 3–12 base pair with nucleotides 86–95 of U6 snRNA: these interactions could result in two closely juxtaposed RNA helices. Such a picture shows at least a superficial similarity with two highly conserved and critical stem-loop structures found in group II self-splicing introns²⁷. On the basis of similar mechanisms, it has been

suggested that nuclear pre-mRNA splicing may have evolved from group II splicing, with snRNAs having a catalytic role in the former process and conserved intron elements performing this function in the latter^{28,29}. The U2–U6 base-pairing interaction may reflect part of a higher-order RNA structure involving snRNAs and the pre-mRNA that is important for catalysing splicing. □

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Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing

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REMOVAL of introns from eukaryotic nuclear messenger RNA precursors is catalysed by a large ribonucleoprotein complex called the spliceosome, which consists of four small nuclear ribonucleoprotein particles (U1, U2, U5, and U4/U6 snRNPs) and auxiliary protein factors^{1,2}. We have begun a genetic analysis of mammalian U2 snRNA by making second-site mutations in a suppressor U2 snRNA³. Here we find that several mutations in the 5' end of U2 (nucleotides 3–8) are deleterious and that one of these can be rescued by compensatory base changes in the 3' end of U6 (nucleotides 92–95). The results demonstrate genetically that the base-pairing interaction between U2 (nucleotides 3–11) and U6 snRNA (nucleotides 87–95), originally proposed on the basis of psoralen photocrosslinking experiments⁴, can influence the efficiency of mRNA splicing in mammals. The U2/U6 interaction in yeast, however, is fairly tolerant to mutation⁵ (D. J. Field and J. D. Friesen, personal communication), emphasizing the potential for facultative RNA interactions within the spliceosome.

Using splice-site competition assays, it has been shown that mutations in the mammalian mRNA branch site consensus (NNYURA*^{*}C, where the asterisk denotes the branch site) can

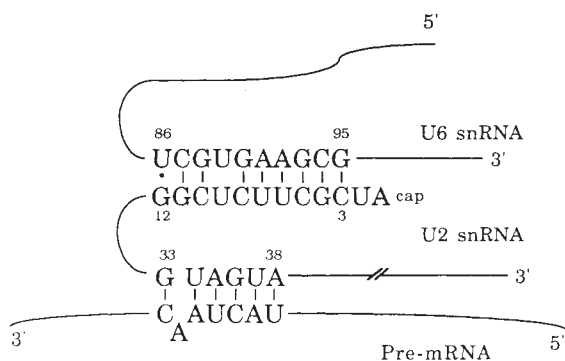


FIG. 4 Diagram of RNA–RNA base-pairing interactions between U2 snRNA and the pre-mRNA and between U2 and U6 snRNAs. Nucleotides 33–38 of U2 snRNA are base-paired with the pre-mRNA branch site, and nucleotides 3–12 are paired with nucleotides 86–95 of U6 snRNA. Note that the pairing involving U2 bases 10–12 and U6 bases 86–88 has not been proven.

TABLE 1 Sequences of mutant U2-35c suppressors and compensatory U6 mutants

		1							11					35					
Wild-type U2	cap	A	U	C	G	C	U	U	C	U	C	G	...	G	U	A	G	U	A
su ⁺ U2-35c	cap	A	U	C	G	C	U	U	C	U	C	G	...	G	U	c	G	U	A
Proposed U2/U6 base pairing	cap	A	U	C	G	C	U	U	C	U	C	G	...	G	U	c	G	U	A
		3'		:	:	:	:	:	:	:	:	:							
				G	C	G	A	A	G	U	G	C	5'						
				95								86							
U2a	cap	A	U	g	a	a	U	U	C	U	C	G	...	G	U	c	G	U	A
U2b	cap	A	U	g	a	a	c	U	C	U	C	G	...	G	U	c	G	U	A
				:	:	:	:	:	:	:	:	:							
U6su ⁺ b		3'		c	u	u	g	A	G	U	G	C	5'						
U2c	cap	A	U	g	a	a	a	U	C	U	C	G	...	G	U	c	G	U	A
				:	:	:	:	:	:	:	:	:							
U6su ⁺ c		3'		c	u	u	u	A	G	U	G	C	5'						
U2d	cap	A	U	g	a	a	g	U	C	U	C	G	...	G	U	c	G	U	A
U2e	cap	A	U	g	a	a	U	c	a	U	C	G	...	G	U	c	G	U	A
U2f	cap	A	U	g	a	a	U	a	C	U	C	G	...	G	U	c	G	U	A
				:	:	:	:	:	:	:	:	:							
U6su ⁺ f		3'		c	u	u	A	u	G	U	G	C	5'						

Wild-type sequences are shown in upper case, mutations in bold lower case. Colons indicate potential base pairs. Ellipses (...) denote U2 sequences not shown. Site-directed mutagenesis²⁷ was performed using an *EcoRI*-*PstI* fragment spanning the human U2-35c gene³ or a *HindIII*-*EcoRI* fragment spanning a human U6 snRNA gene²⁸ subcloned into M13 mp19. Mutations were confirmed by DNA sequencing, and mutagenized snRNA genes were recloned in pUC13 for transfection.

be suppressed by a compensatory base change in the complementary region of U2 snRNA (GUAGUA) (refs 3, 6). To test the significance of a proposed base pairing⁴ between the 5' end of U2 and the 3' end of U6 snRNA, we first asked whether the 5' end of U2 is essential for efficient splicing *in vivo*. Six mutants (Table 1) were made in nucleotides 3–8 of a parent U2-35c construct (A₃₅ to C) which is able to suppress a branch-site mutation in a 3' splice-site competition assay³ using a modified β -globin mRNA precursor designated 10,g, β ¹¹⁰. By making these second-site mutations in a suppressor U2 gene, we were able to perform a genetic analysis of human U2 snRNA structure and function *in vivo* despite the abundance of wild-type U2 snRNA produced by the resident multigene family. All six U2 mutants were intended to weaken the proposed base pairing with U6; three mutants (U2a, b, c) spared the relatively conserved pyrimidines in U2 positions 6–10, but three others (U2d, e, f) did not. The effect of these mutations on U2 function was assayed

as previously described by RNase protection analysis of total cytoplasmic RNA from HeLa cells cotransfected with the U2-35c gene (or mutants therein) and a plasmid construct encoding the 10,g, β ¹¹⁰ mRNA precursor.

Three (U2b, c, f) of the six second-site mutations abolished U2-35c suppressor activity (Fig. 1a, lanes b, c, f) but three did not (Fig. 1a, lanes a, d, e). Failure of mutants a, d, and e to suppress was not due to RNA instability, as observed for certain mammalian U1 mutants⁷; primer extension analysis of total cytoplasmic RNA used in Fig. 1a revealed that expression of all six U2 mutants was comparable to endogenous U6 when the raw data were corrected for a transfection efficiency of approximately 5–10% (Fig. 1b). Thus nucleotides 3–8 of U2 snRNA seem to be required for efficient mammalian mRNA splicing *in vivo*.

We then asked whether the U2 suppressor activity of mutants U2b, c and f could be rescued by making compensatory base

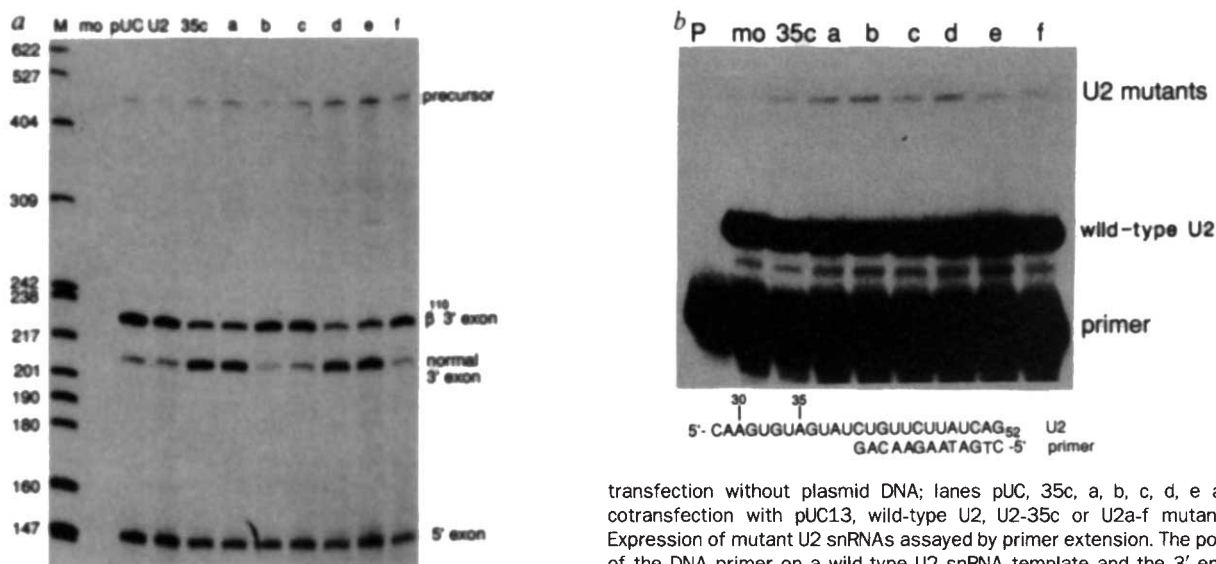


FIG. 1 a, Effect of second site mutations in the suppressor U2-35c snRNA as assayed by RNase protection analysis. HeLa cells were cotransfected with the β -globin 10,g, β ¹¹⁰ mRNA precursor and the suppressor U2-35c or one of six mutants derived from it (U2a–f; see Table 1). M, end-labelled *HpaII* digest of pBR322 (sizes in base pairs indicated to the left); mo, mock

transfection without plasmid DNA; lanes pUC, 35c, a, b, c, d, e and f, cotransfection with pUC13, wild-type U2, U2-35c or U2a–f mutants. b, Expression of mutant U2 snRNAs assayed by primer extension. The position of the DNA primer on a wild-type U2 snRNA template and the 3' ends of the expected extension products derived from wild-type and mutant U2 snRNAs are shown below the autoradiograph. P, primer only.

METHODS. Transfection and assay procedures were as described³, except that a total of 9 μ g plasmid DNA was used per 60-mm plate (4.5 μ g each plasmid). Primer extensions in b were performed³ on the RNA preparations used in a, and the products resolved on a denaturing 15% polyacrylamide gel.

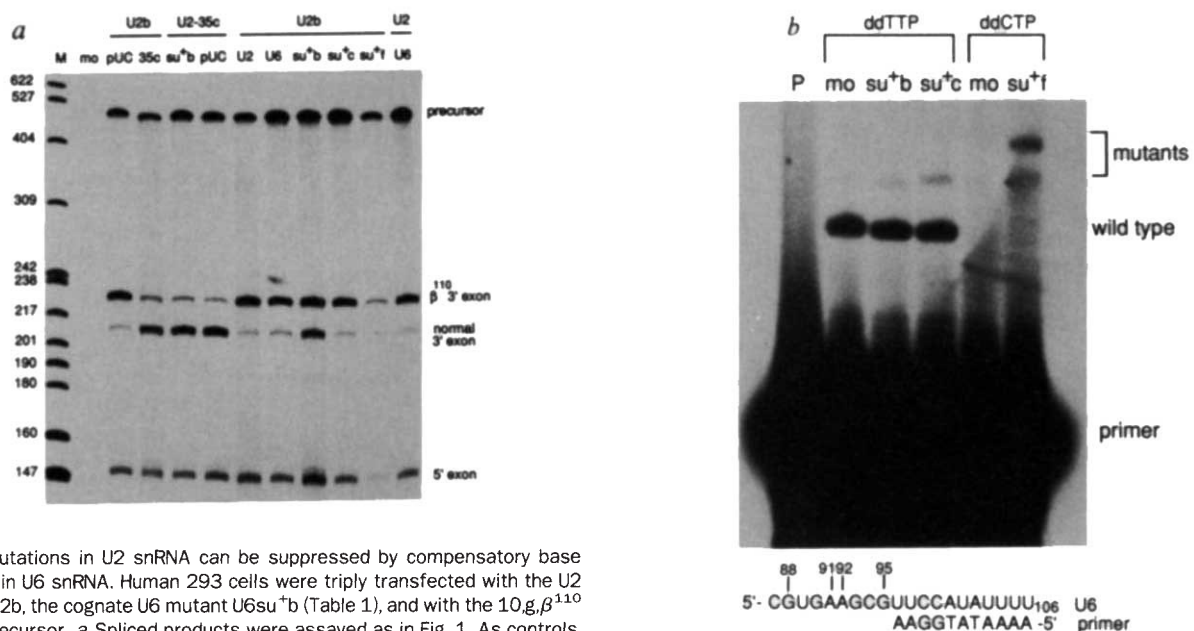


FIG. 2 Mutations in U2 snRNA can be suppressed by compensatory base changes in U6 snRNA. Human 293 cells were triply transfected with the U2 mutant U2b, the cognate U6 mutant U6su⁺b (Table 1), and with the 10.g.β¹¹⁰ mRNA precursor. *a*, Spliced products were assayed as in Fig. 1. As controls, cells were transfected with various combinations of these constructs and wild-type U2, U2-35c, wild-type U6, and pUC13 carrier (see text). M, markers (sizes in base pairs); mo, mock transfection as in Fig. 1. *b*, Expression of mutant U6 snRNAs. Primer extensions on the RNA preparations used in *a* were performed as in Fig. 1b, except that dATP, dGTP, dTTP, and ddCTP were used for U6su⁺f and the corresponding mock-transfected control (mo). P, primer only. The extension products derived from wild-type U6 snRNA (lane mo, in ddCTP panel) and U6su⁺f are obscured because the expected extension products are only one nucleotide longer than the primer itself. The position of the DNA primer on a wild-type U6 snRNA template, and the

3' ends of the expected extension products, are shown below the autoradiograph.

METHODS. For triple transfections, three different plasmid constructs (5 µg each) were used for each 60-mm plate without carrier calf thymus DNA. Transfected cells were lysed for brief sonication followed by treatment of the supernatant with 0.5% SDS and 50 µg ml⁻¹ proteinase K at room temperature for 15 min, and RNase digestion were performed with 50 units ml⁻¹ ribonuclease T1 (Pharmacia) and 10 µg ml⁻¹ ribonuclease A (Sigma).

changes in nucleotides 90–95 of a human U6 gene (mutants U6su⁺b, U6su⁺c, and U6su⁺f; see Table 1). Restoration of U2 suppressor activity was assayed by RNase protection analysis of RNA from human 293 cells cotransfected with pairwise combinations of the U2 and U6 mutants together with the modified β-globin precursor (Fig. 2a). The suppressor activity of U2b was restored by the U6su⁺b extragenic suppressor to 30–50% of the level of the U2-35c parent in several different experiments; moreover, suppression was specific, as U6su⁺c and U6su⁺f had no effect on the phenotype of U2b (Fig. 2a). In contrast, the U2c and U2f mutants could not be rescued by the cognate U6su⁺c and U6su⁺f extragenic suppressors, nor by the noncognate U6su⁺b (data not shown).

Because noncognate U2 suppressors can affect the 3' splice site competition assay (see Fig. 4 in ref. 3), key controls were included to show that the noncognate extragenic suppressors U6su⁺c and U6su⁺f do not restore U2b suppression; that neither U2b nor U6su⁺b interfere with U2-35c activity; that overexpression of wild-type U2 and/or U6 does not restore U2b function or mimic suppressor activity; and that carrier pUC13 plasmid (required to maintain 15 µg DNA per transfection) did not affect U2-35c or U2b expression. These controls confirm that base pairing between U2b snRNA and U6su⁺b snRNA is responsible for suppression.

The failure of the U6su⁺c and U6su⁺f extragenic suppressors to rescue the U2c and U2f mutants cannot be explained by differential stability of mutant U2 or U6 snRNAs in 293 cells. Primer extension analysis of total cytoplasmic RNA used for the RNase protection assay (Fig. 2b, lanes U6su⁺b, U6su⁺c and U6su⁺f; and data not shown) indicated that U6su⁺b and U6su⁺c were expressed at 15–20% of the level of endogenous U6 snRNA, and U6su⁺f at almost twice that level (Fig. 2b). Parallel primer extension analysis on the same RNA showed that U2 mutants U2b, U2c and U2f were equally well expressed in 293 cells (data not shown).

Using a sensitive 5' splice site competition assay, it has been shown that base pairing between U2 and U6 is affected by point mutations⁸. By contrast, our results suggest that base pairing between U2 and U6 can be flexible: U2 mutations (U2e), that seem to disrupt the proposed U2/U6 base pairing severely (Fig. 3) may have no phenotype, whereas U2 mutations that disrupt base pairing equally (compare U2e with U2f, and U2b with U2d) may have different phenotypes. Conceivably, base pairing of G₆ in U2d with A₉₂ in U6 at the end of a short duplex⁹ could explain the difference between U2b and U2d. Finally, although two equally severe mutations (U2b and U2c) both abolish U2 suppression, only one (U2b) of them is suppressible.

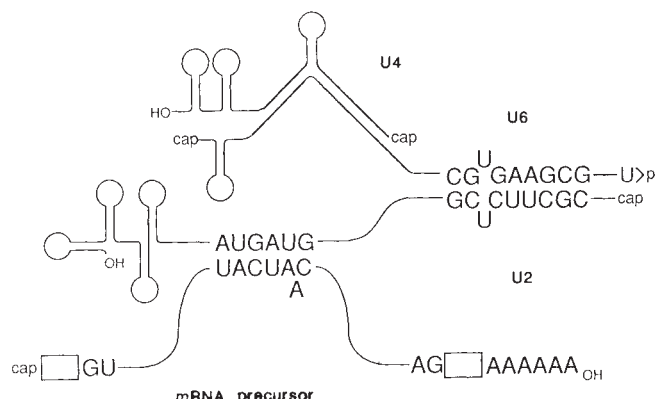


FIG. 3 A model for base pairing between U2 and U6 snRNAs. For clarity, base pairing between U2 and the mRNA precursor is also shown; however, the U2/mRNA and U2/U6 interactions need not occur simultaneously. Although shown as bulged, the U/U opposition in the proposed U2/U6 base pairing is more likely to be intercalated in the manner of a standard base pair²⁹. The 3' end of mature human U6 is shown as a cyclic phosphate (E. Lund and J. E. Dahlberg, manuscript submitted).

The apparently inconsistent behaviour of U2 and U6 mutations in our 3' splice site competition assay defies any simple interpretation, suggesting that the requirement for base pairing between U2 and U6 is not only flexible but subtle. The flexibility of the U2/U6 interaction might also explain, or at least be consistent with, apparently contradictory experimental data regarding the necessity¹⁰⁻¹⁴ or dispensability¹⁵ of the 5' end of U2 in vertebrates, the dispensability of the 5' end of yeast U2 *in vivo*⁵ (D. J. Field and J. D. Friesen, personal communication), and the necessity of the 3' end of U6 for vertebrates *in vivo*^{16,17} but not for yeast *in vitro*¹⁸. Perhaps certain nucleotides are chemically required for catalysis but can be substituted with some loss of activity^{19,20}, or a more complex structure than a standard RNA duplex between U2 and U6 must be formed, or the strength of the U2/U6 interaction must be precisely tuned as is the case for the U4/U6 interaction²⁰. Alternatively, certain mutations could differentially affect localization of the snRNAs *in vivo*.

The proposed U2/U6 base pairs partially overlap the phylogenetically conserved stem I (or A) of U2 snRNA (ref. 21), implying that U2 stem I must melt, partially or completely, to allow base pairing between U2 and U6; strand exchange could occur spontaneously or be promoted by an RNA helicase activity^{22,23}. This reinforces the conclusion, based on disruption of U4/U6 base pairing during the first phosphoester bond transfer²⁴⁻²⁶, that the RNA components of the spliceosome refold in the course of catalysis. Thus, although U4 may indeed repress the catalytic activities attributed to U6 snRNA on the basis of mutational analysis *in vitro*¹⁹ and *in vivo*²⁰, other snRNA components of the spliceosome may contribute to the accuracy or efficiency of splicing. Indeed, the notion of a single catalytic site or snRNA component may be illusory if certain aspects of spliceosome assembly and catalysis cannot be distinguished functionally. Although the U2/U6 interaction does not seem to be required absolutely for catalysis (and is not strictly conserved in either sequence or register⁴), it can affect the overall efficiency of splicing and may play a part in spliceosome assembly, catalysis or disassembly. □

Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network

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THE human papillomaviruses (HPV) are associated specifically with epithelial lesions, ranging from benign warts to invasive carcinoma (for review, see refs 1, 2). The virus encodes three late proteins, which are produced only in terminally differentiating keratinocytes, two of which are structural components of the virion³. The third, E1-E4, is derived primarily from the E4 open reading frame, which represents a region of maximal divergence between different HPV types^{4,5}. E1-E4 does not seem to be a component of the virus particle or to be needed for transformation *in vitro*^{6,7}, but accumulates in the cytoplasm^{5,8-10}, where in certain benign lesions it can comprise 20-30% of total cell protein^{4,10}. We show here that expression of the HPV-16 E1-E4 protein in human keratinocytes (the natural host cell for HPV infection) results in the total collapse of the cytokeratin matrix. Tubulin and actin networks are unaffected by E1-E4, as are the nuclear lamins.

Expression of HPV-1 E4 protein in the upper layers of the skin is correlated with the absence of differentiation-specific keratins¹⁰, and E1-E4 may have a unique function which is specifically related to the epithelial tropism of the virus. To test this hypothesis, a recombinant vaccinia virus was constructed (vacc. E1-E4) and used to infect human keratinocytes, human fibroblasts and monkey CV-1 cells¹¹ (Fig. 1a). The control vaccinia construct, containing HPV-16 L1 in place of E1-E4^{12,13} was used to check infection and expression.

After infection of CV-1 epithelial cells with vacc. E1-E4, the expressed E4 protein localized to a single cytoplasmic inclusion granule 2-5 µm in diameter, adjacent to the nucleus (Fig. 2a). E1-E4 expression was first detected between 5 and 6 h postinfection in CV-1 cells, and between 8 and 9 h in the three keratinocyte lines. These were HaCat, a spontaneously immortalized line which closely resembles normal keratinocytes, both in its cytokeratin expression pattern and its ability to differentiate¹⁴, SVK-14, a simian virus 40 (SV40)-transformed line¹⁵ and ndk, which is restricted for differentiation and resembles basal cells¹⁶. At this stage, E1-E4 was primarily perinuclear but in CV-1 cells it had condensed into inclusion granules by 8 h. This progression was most apparent in the HaCat cell line where the process lasted ≥10 h (Fig. 2e, f, g). The E1-E4 protein was identified in cell extracts as a polypeptide of relative molecular mass 10,000 (M_r , 10 K) (Fig. 1b), in accordance with its predicted size, both from sequence analysis¹⁷ and from *in vitro* transcription/translation studies (ref. 18, and J.D., S.E., M. Hibma, H. Davies and L.C., manuscript in preparation). The E4 antibodies used showed no cross-reactivity with other cellular proteins, except a minor 20K species which was present both in infected and in uninfected cell extracts.

The filamentous appearance of some of the granules at early time points (Fig. 2c) suggested a possible involvement of the cytoskeleton. In epithelial cells, actin, tubulin and the cytokeratins are the main structural components of the microfilament, microtubule and intermediate-filament networks, respectively. Although vacc. E1-E4 infection caused no detectable

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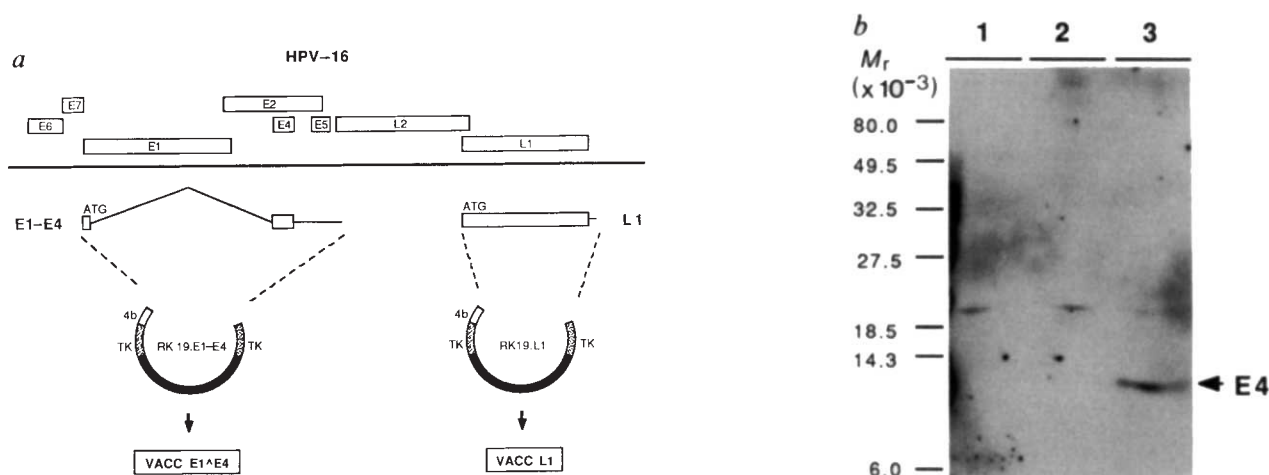


FIG. 1 Structure of expression constructs and detection of E1-E4 protein. *a*, The coding capacity of the E1-E4 and L1 cDNA fragments is shown beneath the complete genome of HPV-16. Coding regions are represented by open boxes with E1-E4 being derived from a spliced transcript. Papillomavirus gene expression was driven by the vaccinia 4b promoter following insertion into the thymidine kinase gene of RK19 and recovery of the vaccinia virus recombinants, vacc. E1-E4 and vacc.L1¹². *b*, Western blot analysis of total protein extracts from uninfected (lane 1), vacc.L1-infected (lane 2) or vacc.E1-E4-infected CV-1 cells using monoclonal antibody TVG 402 (J.D., S.E., M. Hibma, H. Davies and L.C., manuscript in preparation). The position of the E1-E4 protein is arrowed, and M_r markers are indicated on the left. Expression of L1 protein from vacc.L1 has been characterized previously¹².

METHODS. The HPV16 E1-E4 cDNA (ref. 17) was digested to give *Bgl*II cohesive ends and was ligated into the *Bam*HI site of pRK19. Vacc.E1-E4 was subsequently recovered using the WR strain of vaccinia virus, as previously described¹². CV-1 cells grown to 90% confluence in Dulbecco's modified Eagle's Medium (DMEM) containing 5% fetal calf serum (FCS) were infected with recombinant vaccinia virus at a multiplicity of infection (m.o.i.) of 10 p.f.u. per cell, and were harvested 16 h postinfection by solubilization in 5% SDS, 50 mM Tris-Cl, pH 8.0, 1M β -mercaptoethanol. SDS-polyacrylamide gels (20%) were electrophoresed before western blotting using either antibody TVG 401 or 402 (J.D., S.E., M. Hibma, H. Davies and L.C., manuscript in preparation) as described⁵. Blots were developed using the Amersham ECL system.

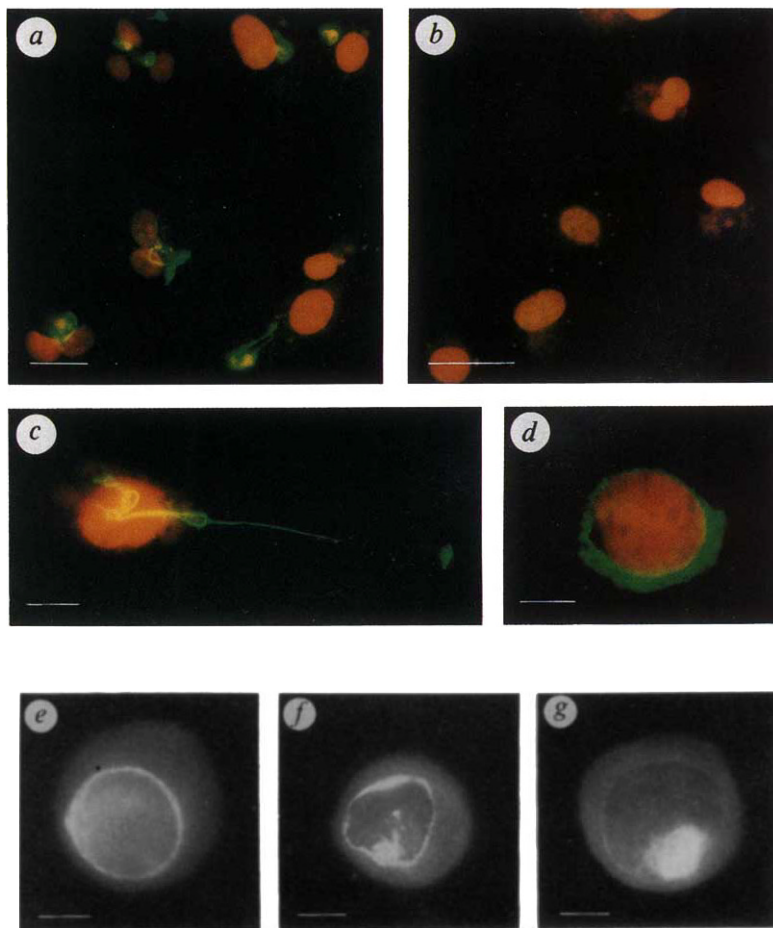


FIG. 2 E1-E4 expression pattern in simple epithelial cells and keratinocytes. *a*, Immunofluorescent staining of vacc.E1-E4-infected CV-1 epithelial cells 15 h postinfection. Inclusion bodies were stained with fluorescein-labelled TVG 402 which is specific for HPV-16 E4 protein (green). Cells were counter-stained with propidium iodide to show the nuclei (red). An identical pattern of staining was observed with polyclonal anti- β -galactosidase E1-E4 fusion protein antiserum, and with serum raised against an E1-E4 N-terminal synthetic peptide (J.D., S.E., M. Hibma, H. Davies and L.C., manuscript in preparation). Scale bar, 10 μ m. *b*, Immunofluorescent staining as in *a*, except that cells were infected with vacc.L1. Neither TVG 402 nor 401 showed any specific staining. Scale bar, 10 μ m. *c*, *d*, E1-E4 staining as in *a* to show the filamentous appearance of inclusion bodies (*c*), and the perinuclear E4 distribution at early times (7 h postinfection; *d*). Scale bars, 2.5 μ m. *e*, *f*, *g*, In the HaCat keratinocyte line E1-E4 formed a perinuclear halo 10 h postinfection (*e*). By 15 h this had condensed (*f*), and by 20 h postinfection E1-E4 was localized primarily to a cytoplasmic inclusion body (*g*). Staining was with TVG 401. Scale bars, 2.5 μ m. E4 antibodies were used at a dilution of 1/25 and propidium iodide at 0.5 μ g ml⁻¹ in the presence of 0.5 μ g ml⁻¹ RNase. Immunofluorescence was essentially as described¹³.

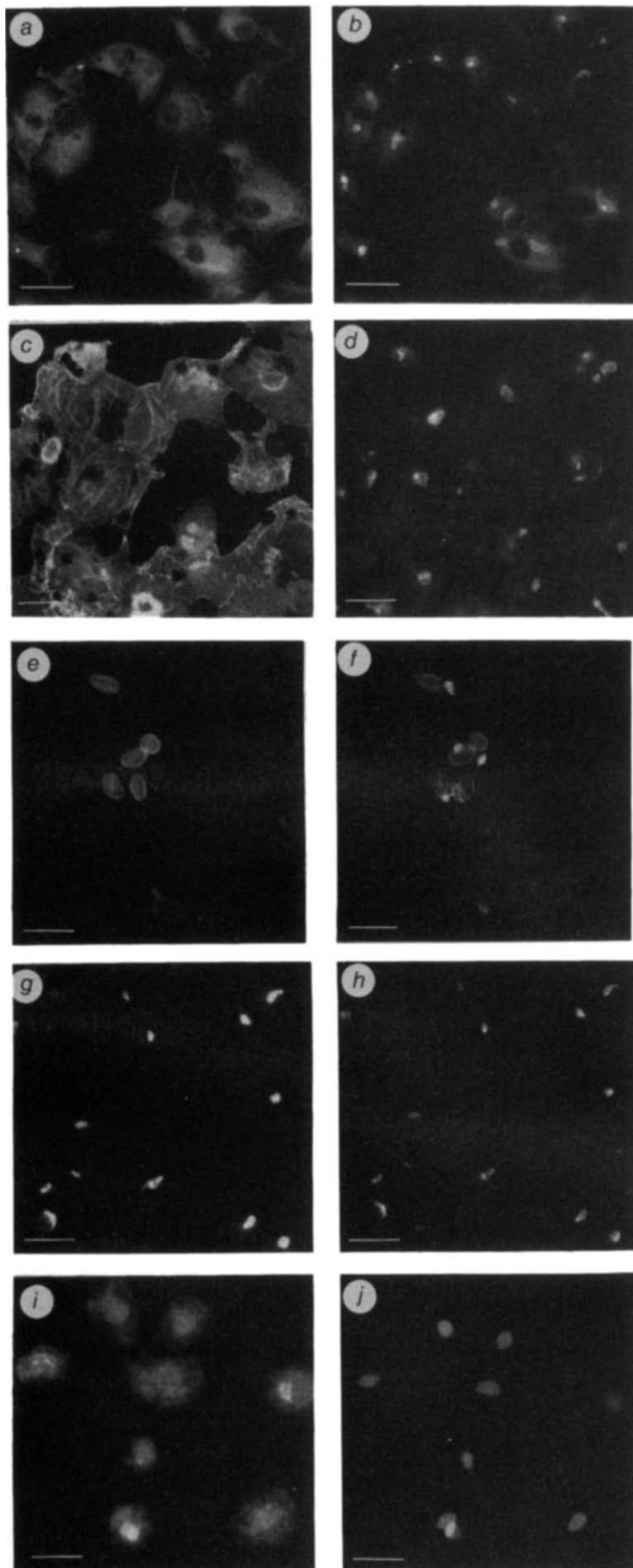


FIG. 3 Association of E1-E4 with the collapsed keratin cytoskeleton. Immunofluorescent staining of vacc.E1-E4-infected CV-1 cells with antibodies to tubulin (a), actin (c, stained using phalloidin-TRITC), lamin B (e) and cytokeratins 8 and 18 (g), is shown on the left, with the corresponding E1-E4 double stains shown in b, d, f and h, respectively, on the right. Cells were infected at a m.o.i. of 10 p.f.u. per cell and fixed after 12 h. i, j, CV-1 cells infected with vacc.L1 at a threefold higher m.o.i., fixed after 16 h and double-stained for cytokeratins 8 and 18 (i) and L1 protein (j). The E1-E4 staining pattern colocalized exactly with that of the cytokeratins, whereas actin, tubulin and lamin networks remained intact. Vaccinia infection alone, even at higher m.o.i., and at a later time postinfection did not significantly damage the cytokeratin matrix. Scale bar, 10 μ m.

METHODS. Cytokeratins were detected using antibodies Cam 5.2 and LP34 (ref. 22). Tubulin antibodies (YL 1/2 and 34) were a gift from J. Kilmartin (LMB, MRC, Cambridge, England) and anti-lamin, A, C (R24) and lamin B (R9) antibodies were provided by G. Krohne (DKFZ, Heidelberg, Germany). Vimentin was detected using monoclonal antibody J144 (provided by J. Kemshead, ICRF, England). Actin was visualized with phalloidin-tetramethylrhodamine (rhodamine) according to the manufacturers recommendations (Cambridge Biosciences, England). For double staining, TVG 402 anti-E1-E4 and Camvir 1 (ref. 13) (anti-L1) were directly conjugated to rhodamine as described²⁷. The first antibody of the double stain was detected using an anti-mouse FITC conjugate¹³ followed by the anti-E1-E4 or the anti-L1 rhodamine conjugate. Cells were either fixed for 10 min in methanol at -20°C (lamin and vimentin staining) or for 5 min in 5% formaldehyde (cytokeratin, tubulin and E4).

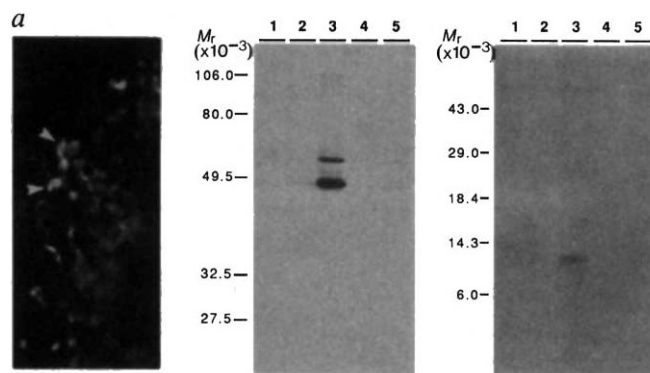


FIG. 4 E1-E4 and cytokeratins copurify during isolation of intact inclusion granules. *a*, Purification of E1-E4 inclusion bodies (arrowed) following retention on TVG 402 (anti-E1-E4)-coated magnetic beads. Inclusion bodies were visualized after purification by incubation with rhodamine-conjugated TVG

change to the actin and tubulin matrices¹⁹ (Fig. 3*a, c*), the cytokeratin distribution was significantly disturbed, forming a filamentous bundle which colocalized exactly with E4 (Fig. 3*g, h*). Although cellular stress can cause intermediate filament disruption²⁰, cytokeratin organization remained apparently normal even 24 h after infection with vacc. L1 (Fig. 3*i, j*). We conclude from this that the HPV-16 E1-E4 protein is specifically involved in causing the collapse of the keratin cytoskeleton and that this occurs irrespective of the particular cytokeratin species present.

A striking analogy can be drawn between this and antibody-induced keratin filament disassembly, which also results in perinuclear filament aggregates²¹. E1-E4 expression caused no disruption to the nuclear lamina (Fig. 3*e, f*), nor to the vimentin network in MRC 5 and W138 human fibroblast lines. In HeLa and TK-143 cells, which express both vimentin and cytokeratin intermediate filaments, the cytokeratins alone became bundled, indicating a specific interaction rather than one which is common to all intermediate filament proteins. The E1-E4-containing aggregates, extracted and purified using antibody coated magnetic beads (Fig. 4*a*), contained both type 1 (K18) and type 2 (K8) cytokeratin (Fig. 4*b*) in ratios similar to those present in uninfected or vacc. L1-infected cells, with no evidence of proteolytic cleavage. Although we do not know whether E4 binds directly to cytokeratins, their colocalization and copurification suggest a close physical interaction.

A cytokeratin filament-collapsing protein would be an ingenious adaptation for a virus which specifically infects epithelial cells. In the skin the keratin cytoskeleton forms a structural scaffold which, through its periodic attachment to the desmosomes, extends right across the epithelial sheet²². If the cytokeratin matrix is just a resistant barrier, then by destroying this, the virus may enhance its chance for release by being shed from the surface of the skin in a fragile squame rather than a highly cornified one⁵. Although cytokeratin expression patterns have not been extensively analysed in tumours, cytokeratins 1 and 10, which are major components of the cornified squame are absent in E4-containing cells of HPV-1-induced lesions¹⁰. The divergence between E4 sequences of different HPV types, which is related to the type of skin each virus infects⁵, may be an adaptation to different cytokeratin expression patterns at different histological sites.

Although we know of no other protein which specifically disrupts keratin filament organization, apart from injected antibodies, it has recently been shown that the adenovirus E1B 19K protein can cause the collapse of vimentin and lamin networks²³

402. Diameter of beads, 4.5 μ m. *b*, Western blot of cytokeratins extracted using antibody-coated magnetic beads (as in *a*) following 10% SDS-polyacrylamide gel electrophoresis and western blotting to Cam 5.2 and LE61 (ref. 22). Lanes 1, 2 and 3 show species extracted using TVG 402 coated beads from uninfected (lane 1), vacc.L1-infected (lane 2), or vacc.E1-E4 infected (lane 3) CV-1 cells. Lanes 4 and 5 show species extracted from vacc. E1-E4-infected cells using Camvir 1 (ref. 13) coated beads (lane 4) or beads coated with antibody 4.37, a HPV-1 anti-E1-E4 monoclonal⁴ (lane 5). M_r markers are indicated on the left. *c*, Western blot of extracts shown in *b* after SDS gel electrophoresis on a 20% gel and western blotting to TVG 402. M_r markers are indicated on the left.

METHODS. CV-1 cells were collected 15 h postinfection and lysed in 0.5% Nonidet P-40 at 4 °C for 10 min as described previously¹¹. Insoluble material was pelleted at low speed (2 min, 6,500 r.p.m., Eppendorf centrifuge) before being resuspended in 500 μ l PBS and mixed with 25 μ l tosyl-activated Dynabeads (Dyna, U.K.) which had been previously coated with protein A-purified antibody and blocked with BSA. Binding and washing were essentially as described for immunoprecipitation¹¹, except that E1-E4 granules were separated from insoluble debris using a magnetic particle concentrator (Dyna, UK). Bound antigen was separated from beads by boiling for 2 min in 9M urea, before being mixed with an equal volume of SDS sample buffer before analysis by SDS-gel electrophoresis.

into an aggregate similar to that shown here with E1-E4. The 19K protein, unlike E1-E4, is known to have a transforming function²⁴ and it has been postulated that intermediate filament damage may alter the organization of the plasma membrane or cytoplasm, so affecting signal transduction and cell growth. It seems likely, however, that the 19K protein and E1-E4 disrupt intermediate filaments for different reasons.

The results presented here add HPV-16 E1-E4 to the diverse group of intermediate filament-associated proteins (see ref. 25), which, with the inclusion of the EBV latent membrane protein²⁶, now contains three virally encoded members. Among these proteins, the cytokeratin specificity of E1-E4 is unique, reflecting a remarkable adaptation of HPV-16 to the epithelial cell it infects. A detailed elucidation of the strategies by which these proteins operate, and the reasons for their existence, will aid our functional understanding of the cellular cytoskeleton. □

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DNA-mediated gene transfer: applications to mosquitoes

Ann M. Fallon

Transfection of cultured cells provides a framework for testing conceptual approaches to genetic manipulation of vector insects.

DNA-mediated gene transfer provides an important tool for exploring genetic, developmental, and regulatory processes that may lead to novel control strategies for pest insects. In particular, current interest in the control of malaria and other mosquito-borne diseases by genetic manipulation of their vectors has been stimulated by the remarkable successes already achieved with the fruit fly, *Drosophila melanogaster*. Molecular approaches with direct bearing on disease control, ranging from disruption of reproduction by expression of antisense RNA molecules¹ to modification of behavior², have been demonstrated in *Drosophila* using transformation vectors based on the transposable element P. Central to the adaptation of this technology to mosquitoes lies the identification and characterization of transposable elements capable of directing the integration of recombinant genes into mosquito chromosomes. Physiological processes that can be manipulated to disrupt disease transmission cycles have yet to be fully defined, and the relevant genes will need to be cloned, analysed, and modified *in vitro* to produce appropriate chimaeric DNA constructs. Once this molecular infrastructure is in place, further challenges remain at the level of the organism, including the development and implementation of strategies for propagating desired traits in natural populations.

Accomplishments to date

Progress towards genetic manipulation of mosquitoes has included modest but encouraging success in transforming embryos using DNA constructs similar to those that have been expressed in *Drosophila*³⁻⁵. Development of mosquito-specific transformation vectors based on endogenous transposable elements such as that recently isolated from the malaria vector, *Anopheles gambiae*⁶, may increase the efficiency with which mosquito embryos can be transformed. At the physiological level, potentially useful phenotypes, such as malaria refractoriness⁷, are beginning to be defined using molecular approaches⁸. Moreover, several groups have begun to clone and characterize mosquito genes from which stage- and tissue-specific regulatory elements can be obtained to engineer gene-

tic constructs specific for disruption of host-pathogen interactions.

Mosquito cell culture

While the technology for efficient expression of foreign genes in mosquito embryos is being developed and perfected, expression of recombinant genes in mosquito cell culture provides an attractive alternative for testing conceptual approaches that eventually can be applied at the level of the organism. Among the numerous mosquito cell lines that have been derived from larval or embryonic tissues, the *Aedes albopictus* line of Singh⁹ has been used most extensively for biochemical and genetic studies. Largely through the efforts of Stollar and co-workers, conditions for mutagenesis¹⁰, selection and analysis of mutants¹¹, and somatic cell fusion¹² have been adapted to these cells, increasing their versatility for molecular studies.

Two protocols for DNA-mediated gene transfer, or transfection, have been developed for *Aedes albopictus* cells. In initial studies¹³, the chemical facilitator polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) was used to introduce the chimaeric plasmid, hsp-CAT, into *Aedes albopictus* cells (see figure). In hsp-CAT, a bacterial chloramphenicol acetyltransferase gene has been placed under the control of the temperature-inducible promoter from

the *Drosophila* heat shock protein (hsp) 70 gene¹⁴. In mosquito cells, both synthesis of endogenous heat shock proteins and induction of CAT expression from the *Drosophila* hsp70 promoter were maximal at 41 °C. Measurable CAT activity could be induced at temperatures as low as 34 °C, however, and conditions for modulating activity from the hsp70 promoter by controlling the temperature and duration of the heat shock have been defined¹⁵. Although genes regulated by weaker promoters, such as that from the *Drosophila* transposable element *copia*, have not been reliably expressed in *Aedes* cells, recently we have detected activity of the promoter in plasmid ptkATO¹⁶, in which the known regulatory elements include only a TATA box from a thymidine kinase gene (C. M. Mazzacano and A. M. Fallon, unpublished data). This development paves the way for using available mosquito cell mutants, such as thymidine kinase-deficient cells¹⁰, as recipients for corresponding selectable genes.

In the genetically-engineered mosquito, stage, sex, and tissue-specific expression of introduced genes ultimately will be important. For the present, however, technological advances will be facilitated by the availability of mosquito promoters that function in cultured cells as well as in any potentially transformed cell in the engineered organism. Promoters from *Aedes* ribosomal protein genes L8 and L31 are thus being sought for the design of vectors that can be universally expressed¹⁷. Later, as experience with the technology is gained, we envision that tissue-specific enhancer elements and alternative promoters may be incorporated to confer appropriate specificity of gene expression. Particularly relevant to disease control, for example, may be regulatory sequences from genes that encode salivary gland proteins¹⁸.

Future prospects

Commercially-available antibodies for detecting hsp-CAT expression allow the researcher to identify transfected cells by histochemical staining. Transfection of *Aedes* cells using polybrene gives a relatively low efficiency of 1 in 10,000 cells¹⁵; however, the method is simple and extremely reproducible. Frequencies as

- DAY 1: Seed 1×10^6 cells per 60-mm dish.
 DAY 2: Examine the cells to ascertain resumption of growth; confluency should be 20–30%. Replace medium with serum-free medium containing polybrene ($12.5 \mu\text{g ml}^{-1}$) and DNA ($6\text{--}12 \mu\text{g ml}^{-1}$). After 6–8 hours, add undiluted DMSO to a final concentration of 25%, mix thoroughly, and incubate the cells for 5 mins at 41 °C. Carefully aspirate the transfection medium and re-feed cells with medium containing serum.
 DAY 4: 48 hours after transfection, incubate the cells under heat-shock conditions (34–41 °C). After a recovery period at the normal growth temperature (28 °C), assay CAT activity in soluble extracts or by histochemical staining.

General protocol for transfecting *Aedes albopictus* cells with hsp-CAT DNA using polybrene as the chemical facilitator. For further details, see refs 15 and 19.

high as 1 in 1,000 can be obtained when mosquito cells are transfected with DNA-calcium phosphate co-precipitates, but the procedure requires substantially greater attention to the condition of the cells and to the precision with which the co-precipitates are prepared¹⁹. To facilitate recovery of transfected cells in quantities sufficient for molecular analysis of recombinant gene products, present efforts focus on developing vectors containing genes that function as dominant selectable markers in mosquito cells.

Early attempts to transfect thymidine kinase genes into thymidine kinase-deficient mosquito cells yielded clones that had become resistant to methotrexate in the selection medium. Derivatives of these resistant cells provided an amplified dihydrofolate reductase (DHFR) gene, which now has been cloned. Fortuitously, the mosquito gene lacked the large introns (up to 16 kb) that are characteristic of vertebrate DHFR genes, facilitating recovery of the entire gene (with a single 56-nucleotide intron near the 5'-end) on a single 1.8 kb *AccI* fragment²⁰. By introducing the mosquito DHFR gene with its own promoter into the hsp-CAT plasmid, a DNA construct was obtained whose retention can be ensured by applying methotrexate selection after transfection. The heat-inducible CAT activity encoded on the same molecule facilitates rapid histochemical or biochemical verification of plasmid retention. Ongoing studies suggest this plasmid is stably maintained in mosquito cells (F. A. Shotkoski and A. M. Fallon, unpublished data). As has been demonstrated already with vertebrate cells²¹, site-directed mutagenesis may enhance the versatility of vectors containing the mosquito DHFR gene.

Use of selectable genes to recover clonal populations in which all cells maintain transfected DNA will facilitate characterization of chimaeric gene products, analysis of non-inducible or weak promoters and optimization of vectors for specific applications. In the transgenic mosquito, for example, disruption of pathogen biology may require recombinant genes whose products are secreted into the haemolymph. Cultured cells provide a simple system for assaying the efficiency of signal peptides and other regulatory elements, so that only those candidates with a strong likelihood of conferring the desired phenotype are used for the more labour-intensive injection of mosquito embryos. Similarly, analysis of the synthesis and function of transposases in cell culture will facilitate attempts to modify the *Drosophila* P element for expression in mosquito cells, or to evaluate endogenous mosquito transposable elements as they become available. Judicious integration of cell

Cell culture supplement

A new substrate for the growth of anchorage-dependent cells and a closed perfusion chamber that provides the right environment for making single-cell fluorescence measurements — new tools and tips for cell and tissue culture.

BIOGENEX Laboratories has developed a novel Antigen Retrieval System that can be used to **recover antigenicity in formalin-fixed, paraffin-embedded tissue**, without the use of enzyme predigestion (*Reader Service No. 101*). The method involves the microwave heating (to over 100 °C) of tissue sections in the presence of the company's specially formulated solution. Using this method, BioGenex claims that positive immunostaining has been demonstrated in tissues that have been fixed in formalin for over two years. In addition, this method has been shown to enhance or recover full staining with over 70 per cent of the antibodies tested to date, including several that previously were unreactive with formalin-fixed tissue. Antigen retrieval has been shown to increase staining intensity or reduce background staining of many important clinical markers, such as cytokeratin and vimentin, BioGenex says. In addition, it should reduce false negative staining of overfixed tissue, expand the range of antibodies useful for routinely processed tissue, and increase the usefulness of archival materials for retrospective studies. The Antigen Retrieval System contains 250 ml of 4× concentrated antigen retrieval solution and the complete retrieval protocol. The protocol can not be used to rescue alcohol-fixed or frozen tissue sections.

Plastic cultureware precoated with Extracellular Matrix (ECM), a **substrate for**

the growth of anchorage-dependent cells, is now available from Baxter (*Reader Service No. 102*). ECM is a natural matrix produced by bovine corneal endothelium cells, which has a similar organization and chemistry to basement membranes. Baxter says that ECM can provide higher cell proliferation rates and greater responsiveness of cells to growth factors and hormones than cells grown on uncoated cultureware. It could prove useful in the study of neoplastic growth and invasion, cell attachment and viral invasion. ECM-coated plates are available as a three-pack of either 24-well or 96-well plates. Each plate is supplied individually foil-wrapped.

Marrow-Tech has developed Skin² **neutral red cytotoxicity assay kits**, which are designed to enhance the accuracy and reproducibility of *in vitro* dermal or ocular cytotoxicity screening when used in conjunction with the company's three-dimensional Skin² tissue substrates (*Reader Service No. 103*). Skin² yields mechanistic information on how test agents affect the lysosomal activity of living cells. It can be used to predict the human cytotoxic effects of existing, new or reformulated products, Marrow-Tech says. Each Skin² neutral red assay kit is designed to be used with one 24-well Skin² ("skin-squared") toxicity screening kit, which features living, three-dimensional human skin tissues that are grown from individual skin cells *in vitro*.

culture approaches with transformation efforts at the level of the organism will improve prospects for control of mosquito-borne disease by genetic manipulation of its vectors. □

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using the company's patented core technology. The Skin² neutral red assay kit includes all the reagents necessary to generate statistically valid dose-response curves for two different test compounds (at five dilutions, each in duplicate), the company says.

Odds and ends

Over 100 new strains are listed in the latest edition of the *ATCC Catalogue of Protists* from the American Type Culture Collection (*Reader Service No. 104*). Significant new additions to the **protist catalogue** include all 23 WHO reference strains of *Leishmania*, 11 strains of *Trichomonas vaginalis* for use in *in vitro* testing of metronidazole susceptibility, an *in vitro* and an *in vivo* strain of *Toxoplasma gondii*, one strain of *Blastocystis hominis* and several axenic strains of marine Scuticociliates. The catalogue provides complete strain descriptions including history, important references citing the strain, mode of shipment, and detailed methods for handling certain strains. In addition, the catalogue includes details of media formulations used in the cultivation of the listed algal and protozoan strains.

To promote good laboratory practices when using biological safety cabinets, the Eagleson Institute of Sanford, Maine has produced a new **lab safety videotape** (*Reader Service No. 105*). Entitled "Safe Use of Biological Safety Cabinets or The Case of the Contaminated Cultures", the video is a dramatisation of a typical laboratory where cultures have become

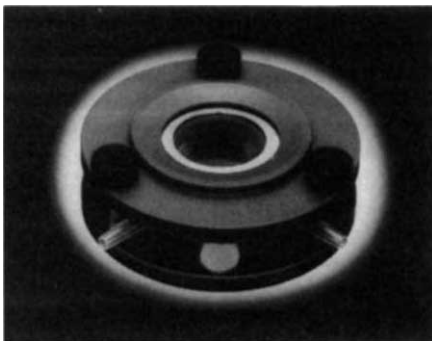


Sleuthing out the contamination source.

contaminated. The ensuing investigation into the source of the contamination prompts the researchers to evaluate their technical procedures. The investigation offers information on how safety cabinets work, rules and guidelines for work preparation, procedures for clean-up, and tips on how to improve work habits to maximize safety.

Benchtop gadgetry

The LU-CPC closed perfusion chamber, which was developed at the University of Leiden, The Netherlands and is available from Medical Systems, is designed for use on the stage of upright or inverted microscopes (*Reader Service No. 106*). The chamber is intended to be a useful tool in



Closed perfusion chamber.

the measurement of calcium concentration, pH or membrane potential using fluorescent dyes under physiological conditions. It can also be used where perfusion and good optical access are required. The system is made up of standard circular glass coverslips (24/25 mm diameter), which are attached by snap-type fittings. Cells are grown on the coverslips and are attached to the top or bottom of the chamber depending on the type of microscope used. Short optical paths between the coverslips (14 mm) allow fluorescence, phase contrast, interference contrast and confocal microscopy to be performed, the company says. Eight conically-drilled holes in the side of the LU-CPC chamber permit the insertion of perfusion lines, probes, and a thin, spiral stainless steel tube to control the chamber environment when perfusion is stopped.

With the introduction of the new Cytospulse 901 **electrofusion device** from Quantum Biosystems, the company claims that many of the problems associated with the generation of rodent and human hybridomas have been ironed out (*Reader Service No. 107*). When using the integral flow cell, which provides a temperature-controlled pulsing environment, cell fusion occurs under physiological conditions. According to Quantum Biosystems, this represents a considerable improvement over existing technology. Other instruments require low ionic strength solutions, such as mannitol and sucrose, to prevent heating induced by electrical currents, which can perturb the 'pearl chain' formation necessary for cell fusion by conduction effects. The cells to be fused pass slowly through a water-cooled capillary and are subjected to direct current pulses via electrodes of salt solutions, separated by semi-permeable membranes. This avoids the problems

associated with conventional metallic electrodes, such as cell aggregation and mortality caused by 'gassing' effects, the company says. Flow and pulse rates, which are under microprocessor control, are pre-set by the operator to optimize run conditions for specific applications and cell types. Fused cells are collected for subsequent *in vitro* culture. All procedures are carried out aseptically and the entire flow cell can be dismantled for cleaning and autoclaving. The instrument can also be used as an electroporation device for genetic manipulation.

Launched three months ago, the Select Analyst HP **self-contained water purifier** from Purite is designed primarily for applications, such as HPLC, fluorescence analysis, ion chromatography and tissue culture, where a water source that is virtually free of metal ions, bacteria, pyrogens and organics is required (*Reader Service No. 108*). The Select Analyst HP embraces the technologies of reverse osmosis, nuclear ion exchange, organic removal and sub-micrometre particulate filtration in a compact wall- or bench-mounted unit. Other design features include a 25 litre storage tank, a recirculation pump that ensures there is no stagnant water within the system, and a tap for drawing off water immediately after it has been purified. Three models are available with outputs of 2, 4 and 8 litres per hour, costing £1,430, £1,590 and £1,780 (UK), respectively.

Media medley

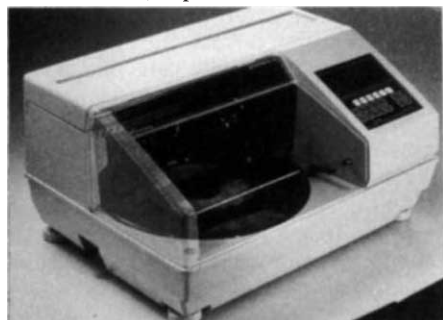
Gibco-BRL has added two new products to its line of **serum-free media** (SFM): Hybridoma-SFM and Macrophage-SFM (*Reader Service No. 109*). Hybridoma-SFM allows researchers to avoid the complications of growing hybridomas in conventional basal media. It is a low protein (20 µg ml⁻¹), defined medium for serum-free growth and maintenance of a wide variety of hybridomas. According to Gibco-BRL, hybridoma growth and monoclonal antibody production are enhanced when using this product, when compared to serum-supplemented or other serum-free formulations. Macrophage-SFM was developed for the culture of human peripheral-blood monocytes and macrophages, one of the initial cell types infected with HIV-1. The medium can support the long-term maintenance of human macrophages for use in adoptive immunotherapy and AIDS research. And, as Macrophage-SFM is serum-free, it not only provides a low background for the detection of cellular products, but also eliminates the risk of contamination from adventitious viruses often associated with serum-supplemented media. Gibco-BRL states that Macrophage-SFM has a specification of less than 0.1 ng ml⁻¹ endotoxin.

PENTEX EX-CYTE VLE, a **growth enhancement media supplement** from Miles, is designed to offer all the advantages of serum-free media without surrendering cell growth and product yield (*Reader Service No. 110*). The product provides a serum-free media with a natural balance of cholesterol, phospholipids, triglycerides and essential fatty acids. As these are bound to their natural lipoprotein carriers, EX-CYTE VLE is water soluble and easily metabolized by cells. It is free of immunoglobulins, organic solvents or detergents, is certified free of viruses and mycoplasma, and is very low in endotoxin, Miles says. It can be used in conjunction with other PENTEX components for serum-free media, such as transferrin, insulin, albumin, fetuin and egg-yolk extract.

Three human **interleukin-2 (Il-2)** preparations for research use are now available from Boehringer (*Reader Service No. 111*). The line-up includes: natural Il-2 from human T lymphocytes, recombinant human Il-2 from *Escherichia coli*, and cell-line derived natural Il-2 prepared from the supernatant of an established cell line. The recombinant human Il-2, which is isolated from *E. coli* (gene cloned into pBR322), is free from other lymphokines, interferons and lectins, Boehringer says. The company claims that its preparations offer the lowest recommended working concentrations, in addition to long product stability and the convenience of high (10,000 units per ml) and low (200 units per ml) concentrations. To ensure product consistency, each lot is quality-control tested for product performance and bioassayed against biological response modifiers programme standards to check unit activity. Il-2, also known as T-cell growth factor, can be used to cultivate and proliferate HIV-positive T-cells, sustain primary cultures or peripheral-blood mononuclear cells, and maintain T-cell co-cultures.

Slide stainers

Automate your routine immunohistochemical tissue staining methods with the Jung Histostainer Ig from Leica (*Reader Service No. 112*). Using the **automated slide stainer**, up to 20 slides can be



Leica's automated slide stainer saves time and cuts operating costs.

processed simultaneously, each with any 10 programmes using the operator's own choice of reagents. The instrument is programmed via a microprocessor-controlled, menu-driven operating system. To minimize the amount of immunoreagents used, a patented spray system provides precise delivery of reagents to a selected area of the slide, Leica says. The slides fit on a carousel and are processed in a humidity-controlled chamber to prevent drying during lengthy staining protocols. In addition, temperatures of between ambient and 45 °C can be preselected to enhance and speed up the staining procedure. Operators can also choose whether to dispense expensive primary antibodies or chromagen substrates manually or automatically. An optional battery back-up is available in the event of power failure.

Carl Zeiss offers four **programmable slide stainer modules** in the HMS Series, ranging from six to 22 stations and offering either a 50- or 100-slide capacity (*Reader Service No. 113*). The units are designed to be easy to programme and offer many more options than simply setting up the stations in a direct line: reagents and stains can be grouped and the robot arm can be directed to each station in the order and for the length of time required. Up to nine user-defined programmes, of up to fifty steps each, can be stored in the instrument's memory. To eliminate water damage to electronic functions, all key electronic components are elevated above the staining stations in a pyramid-shaped design. In addition, stainless steel trays and a 'shake' function help maintain cleanliness and reduce contamination of reagents from one bath to the next. For safety purposes, the unit is fitted with a hood, to contain fumes, and an extractor fan. An optional vent tube kit is available for venting fumes to an external hood or charcoal filter. Each unit comes complete with an integrated cover, washing and drying tanks, a bucket carrier, reagent buckets, a slide basket and a basket tool. The modular design of the slide stainers allows you to upgrade to a greater number of reagent buckets (from single-sided to double-sided) as your requirements change.

Cell culture systems

Cell-Pharm IV, the latest **hollow fibre bioreactor system** from UniSyn Fibertec, is intended for the large-scale production of mammalian cell culture products such as monoclonal antibodies, recombinant proteins, viruses and viral antigens, or other secreted proteins (*Reader Service No. 114*). The system incorporates four large (3.2 square metre) bioreactors, which are capable of routinely producing monoclonal antibodies in excess of 75 grams per month at cell densities of



Cell-Pharm IV bioreactor uses hollow-fibre cell culture technology.

greater than 10⁸ cells per millilitre. Improved process monitoring and control of pH, differential dissolved oxygen, temperature, recirculation rates and product harvest collection, and a complete software package for data logging and analysis, graphing, multi-tasking and networking are additional features of the system. For anchorage-dependent cell lines, UniSyn Fibertec has introduced a new 2.0 square metre ampholytic copolymer membrane bioreactor, which is designed to yield up to ten times more product than other membranes. A reduced cell-growth lag phase produces shorter production runs, the company says.

The Caro-CRL **compact laboratory fermenter** from Caro Biotechnik, with total volumes of either 3, 5, or 10 litres, is intended for batch, continuous, aerobic



Caro Biotechnik's compact lab fermenter.

and anaerobic processing (*Reader Service No. 115*). The basic fermentation system, which costs about DM18,000 (Germany), consists of a 5-litre stirred vessel, temperature control device, aeration device, adjustable drive motor, pH control device, substrate pump and dosing pumps. Standard size ports of 8–12 mm and 10–18 mm permit the installation of feed and outlet lines, air input, air output and probes. The complete system can be sterilized in a benchtop autoclave.

Frozen assets

The new Cryo 1 °C **freezing container** from Nalge is designed to meet the needs of smaller laboratories with low-volume cryopreservation requirements (*Reader Service No. 116*). The container, which

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Reader Service No.57



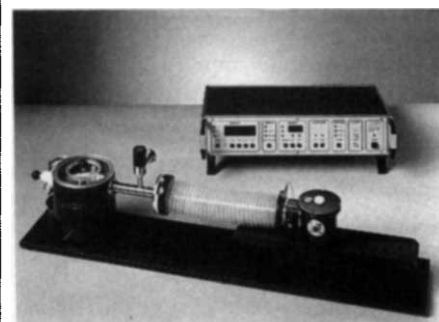
Nalge's Cryo 1 °C freezing container.

holds 18 Nalgene 1.2- and 2-ml cryovials or other vials of the same dimensions, provides the critical -1°C cooling rate that is required for successful cell cryopreservation and recovery, Nalge says. In addition to the container, all that is required is isopropyl alcohol and a mechanical freezer. To prevent contact with the alcohol and contamination by 'wicking', the vials are held in a rigid, high-density polyethylene holder, which can be removed from the container and placed in a water bath to thaw the contents of the vials. All components of the container are designed to withstand repeated freezing/thawing cycles. Whether empty or full, the containers can be stacked.

Finn-Aqua's Lyovac GT 2 freeze-drying unit is designed to offer optimum processing conditions for condensing products that are sensitive to temperature (Reader Service No. 117). The unit can be used for drying samples in flasks, ampoules, vials or on trays. Varied and versatile accessories permit freeze drying at the manifold plate or in trays within the chamber. The Lyovac GT 2 is designed around the two-chamber principle with spatial separation of the drying and condensing areas. This allows pressure rise measurements to be carried in the drying chamber so that an accurate determination of the end of the drying process can be made, Finn-Aqua says. Because of its modular design, the Lyovac GT 2 can be fitted with a stoppering device to permit stoppering of vials on one or several shelves. Ease of operation was a high priority, Finn-Aqua says, and so the Lyovac GT 2 features automatic process control and monitoring using a microprocessor-controlled module, touch

panel and large LCD, together with a facility for rapid defrosting of the condenser under visual control.

For the frost-free transfer of both specimen and holder, Oxford Instruments has introduced the new CT3500 cryo-transfer system for transmission electron microscopes (TEMs) (Reader Service No. 118). Cryo-transfer for transmission electron microscopy offers several advantages over conventional specimen preparation techniques, the company says. First, hydrated or low melting point materials such as biological cell suspensions and frozen sections can be examined. Second, it allows X-ray mapping of unfixed cryosections. This allows detection of ionic concentration, such as calcium and sodium ions. And third, following sample preparation and coating, cryo-transfer will allow cryo-SEM (scanning electron microscopy) to



CT3500 for frost-free cryo-transfer.

be performed in the STEM (scanning transmission electron microscope). Oxford Instruments claims that the CT3500 nitrogen cooling model offers 0.34-nm performance, even at tilt angles of up to 45° . The side-entry TEM cryo-transfer holder has been designed to produce very stable cryogenic temperatures for long periods with negligible drift and vibration. 'Double-shield' technology incorporated in the CT3500 means that immediate examination of the specimen is possible. The specimen itself is protected and kept cool during transfer by an externally controlled cooled shutter, which shields both above and below the insert. Retractable bellows purged with cold dry nitrogen gas protect the specimen holder tip from frost contamination, allowing an uninterrupted succession of transfers to be carried out. The system comes complete with an 'intelligent' three-term temperature controller, nitrogen heat exchanger, an integrated workstation and a protective shroud for the holder tip. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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